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CYTOLOGY AND CYTOCHEMISTRY OF MELANOMA CELLS

By

SYLVIA S. GREENBERG, M. J. KOPAC, AND MYRON GORDON

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CYTOLOGY AND CYTOCHEMISTRY OF MELANOMA CELLS*

By

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Introduction

The spontaneous pigmented neoplasms that develop in platyfish-swordtail hybrids (*Xiphophorus maculatus* × *Xiphophorus helleri*) are referable to specific genes and their modifiers.[1] To obtain an insight into the mode of action of the genetic factors involved in the atypical pigment cell growth, the cytological and cytochemical properties of the tumors were studied. The analysis was applied to those cells that proliferated in tissue culture from 4 color variations of melanotic tumors. These included (1) typical black melanomas, (2) melanomas with nonpigmented areas, (3) amelanotic melanomas from albino hybrids, and (4) amelanotic melanomas with pigmented areas. The manifestations of the albino gene in amelanotic melanomas were investigated, and an analysis was made of the pigment contained in some of the tumors of homozygous-albino hybrids.

Materials and Methods

Platyfish-swordtail hybrids with large, well-developed tumors, presumably in the terminal stages, were selected as far as possible. The tumors were situated in different regions of the body as follows: (1) melanomas of the dorsal fin, influenced by the spotted dorsal gene, *Sd,* and its modifiers; (2) melanomas on the flanks, influenced by the striped-sided gene, *Sr,* and modifiers; (3) melanomas on the ventral part of the body that are referable to the spotted-belly gene, *Sb,* and modifiers; (4) caudal peduncular amelanotic melanomas in albino hybrids involving the black-banded (nigra) gene, *N,* modifiers, and the recessive albino gene, *i*; and (5) dorsal fin amelanotic melanomas involving *Sd,* modifiers, and the albino gene.

The tumors were excised from the living animal and disinfected in an aqueous merthiolate solution, diluted 1:10,000 with sterile Holtfreter's solution for 2 to 5 minutes.[2] It was not possible to obtain sterile tissue since the epidermis was not removed. The epidermis was cultured together with the underlying tumor because of its hyperplastic nature and involvement in most of the growths.[3] The tumors were washed in 2 changes of sterile Holtfreter's solution containing 100 units of penicillin G and 0.01 mg. streptomycin hydrochloride per cc. The tissue was kept in the last solution for approximately 30 minutes, and then cut into fragments measuring 1 to 2 mm. The Maximow double-cover slip method of tissue culture was used as described by Cameron.[4]

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*From a thesis presented to the Faculty of the Graduate School of Arts and Science, New York University, by Sylvia S. Greenberg, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Two different combinations of culture media were compared. The first consisted of 1 part fowl plasma, 1 part chick-embryo extract, and 3 parts homologous fish serum, as recommended by Grand, Gordon, and Cameron.[2] The fish serum was obtained from a carp, under sterile conditions, by means of a cardiac puncture with a hypodermic needle. The blood was centrifuged in a sterile tube and the serum pipetted off. In the second mixture, the carp serum was replaced by 2½ parts of filter-sterilized human ascitic fluid. The latter was finally selected and used throughout this investigation, as cell growth was faster and more luxurious than with the carp serum.

The cultures were examined daily with the microscope, and their growth and development was recorded by means of photomicrographs. A total of 712 cultures, obtained from 18 tumors, was studied.

CYTOLOGICAL PROCEDURES

The cultures were fixed, stained, and analyzed as whole mounts. Every cell in the outgrowth zone was observed completely with all its processes and ramifications, and with its relationship to the explant mass. For general morphological analysis, the cultures were fixed in Bouin's fluid, and stained with Harris' hematoxylin and eosin.

CYTOCHEMICAL PROCEDURES

Various cytochemical techniques were used for the *in situ* localization of organic entities. Since these methods have certain limitations, with reference to their sensitivity and diffusion, more than one method was used wherever possible. In correlating the results obtained with the various techniques, the significance and limitations of the individual tests were kept in mind.

Nucleic Acids

Desoxyribonucleic acid (DNA) was studied by means of a modified Feulgen technique.[5,6] For the demonstration of ribonucleic acid (RNA), pyronin B was used. The relationship of both types of nucleic acid in the cell was demonstrated with methyl green and pyronin by the methods of Brachet,[7] Kay,[8] Kurnick,[9-11] Pollister and Leuchtenberger,[12] and Taft.[13] The May-Grunwald-Giemsa stain also demonstrated the two types of nucleic acid.[14,15] A rough quantitative estimate of DNA was made by extraction with hot trichloracetic acid for different lengths of time.[16]

Polysaccharides

Glycogen was demonstrated with periodic acid and Schiff's reagent.[17] Controls were subjected to salivary digestion prior to the glycogen technique. Other polysaccharide tests that were used in an attempt to identify various cellular constituents include toluidine blue staining,[18] mucicarmine,[19] alcian blue,[20] rose bengal,[21] and the silver technique for glycogen and mucin.[17]

Lipids

To localize lipids and lipid complexes, the following methods were employed: (1) the plasmal and pseudoplasmal reactions of Cain,[22,23] Dañielli,[24,25] and Deane and Andrews;[26] (2) the Sudan IV, Sudan black B, and Nile blue A stains;[17] (3) Menschik's[27] Nile blue method for phospholipids, and Baker's[28] acid hematin test for phospholipids; (4) Ackerman's technique[29] for masked lipids; (5) lipid extraction techniques.[17]

Thiol Groups

Two methods were employed for the detection and localization of thiol groups: (1) Chèvremont and Fredéric's ferricyanide method;[17] and (2) the more specific method of Barrnett and Seligman.[35] With both methods, controls were used that were inhibited with iodoacetic acid prior to the reaction.

Melanin Reactions

The silver reduction or argentaffin techniques of Masson-Fontana[17] and Tandler[30] were used for the demonstration of melanin. Silver impregnation was carried out using Cajal's technique.[21] The bleaching of melanin was demonstrated with the peroxide, permanganate, chromic acid, and nitric acid methods.[17] For the enzymes involved in melanin formation, the dopa and tyrosine reactions were used.[31-33] Controls were subjected to inhibitors of tyrosinase activity.[33,34]

Other Reactions

The 5-day osmium tetroxide technique of Worley and Spater[36] was used to demonstrate reducing groups. With the performic acid-Schiff technique,[17] disulfide linkages were localized. A modification of the Gomori-Takamatsu technique was used for the demonstration of phosphatase activity.[18]

Terminology

The pigment cell terminology recommended at the Third Conference on the *Biology of Normal and Atypical Pigment Cell Growth*[37,38] is followed in this paper. The *melanoblast* is the embryonic, nonpigmented, pigment cell. It becomes a *melanocyte* when melanin appears, and is characterized by its thin dendritic processes. The *melanophore* is a pigment effector cell usually found only in the lower vertebrates. The *macrophage* is a phagocytic cell that may contain engulfed melanin in irregular clumps.

A stage of the pigment cell, between the melanoblast and melanocyte, was encountered frequently in the cultures of fish melanomas. It resembled the melanocyte except for the granules, which were colorless rather than pigmented. Consequently, this cell has been described as a young melanocyte.

Results

MELANOMAS

Gross Morphology

Macroscopic inspection of the melanomas in several animals revealed differences in texture, color, and degree of infiltration of the subcutaneous tissues. The tumors were either single, large masses with smooth, regular surfaces, or they consisted of several nodular masses, which gave the overgrowths a lobulated appearance. When scales were present, they were raised and separated, probably due to pressure from the growing tumor below. In most of the overgrowths, however, the scales were completely destroyed. On dissecting the tissue, the tumors showed variable resistance to cutting. Some were firm or definitely hard and fibrous; others were ulcerated, soft, and gelatinous and were impossible to cut evenly. Frequently, different areas of a single tumor varied in consistency. Tumors that had destroyed and replaced fins contained remnants of the fin ray cartilage.

Many of the overgrowths were not uniformly black but contained limited areas of gray or white. Over-all, grayish-black tumors were also observed. The white areas were either translucent or opaque. On dissecting the all-black tumors, they revealed 2 different distributions of pigment: 1 type was black throughout; in the other, the black pigment was limited to a thin surface coating, while the inner mass was white. Two discrete tumors were often present in a single animal, each exhibiting a different texture and pigment pattern; the distance between them usually was small.

Growth Patterns and Cell Types in Tissue Culture

In all actively growing cultures, the plasma clots were liquefied on the first day of growth, and became progressively more so with time.

Small tumors, with as yet no serious effect on the body economy, formed scanty outgrowth. On the other hand, large, well-advanced tumors, taken from animals that showed definite signs of distress, grew luxuriantly in tissue culture. Observations on the proliferative ability of the tumors are in agreement with those of Grand, Gordon, and Cameron,[2] who compared melanotic fish tissue in the early stages of melanosis with definitive melanomas. The hard, fibrous tumors grew very little or not at all. The softer, edematous ones grew luxuriantly.

The cell types that developed from the melanoma tissue included melanocytes, melanophores, macrophages, fibrocytes, and epithelial cells.

Melanocytes. The preponderant cell type in tissue cultures was the melanocyte. It had 1 to 5 oval to round nuclei. The majority of cells, however, were binucleate. Each nucleus had 1 to 3 small, round nucleoli, but occasionally a single, lobulated nucleolus was found. This may represent the incomplete fusion of several nucleoli, or a single large nucleolus breaking down into smaller bodies.

The dendritic processes of the melanocytes emerged at the margin of the fragment on the first day *in vitro* (FIGURE 1). A few whole cells were also present, but the majority of cell bodies usually did not appear until the third or fourth day. Melanocytes varied in size, shape, and degree of pigmentation. The smaller cells had fine, uniformly distributed black or brown granules. The dendritic processes were thin and delicate (FIGURE 2). The melanocytes that developed from ventral region tumors of spotted-belly (*Sb*) hybrids were usually of the small type, with fine, powder-like, brown pigment granules. Large melanocytes had coarser granules that were unmistakably black (FIGURE 3). The processes were thick and heavily laden with melanin. The large melanocyte was the prevalent cell type in cultures from a flank tumor of a striped-sided *(Sr)* hybrid. Between these extreme types were melanocytes with finely granular cell bodies and clumps of melanin at the tips of their dendrites, which gave them a knobbed appearance (FIGURE 4). Frequently the knobs became detached and remained free in the medium. Whole dendritic processes also became knobbed and beaded with black swellings and constrictions along their entire length. These were pinched off at the base, close to the cell body, and remained free in the medium. Grand[39] reported knobbed fragments in cultures of mouse melanoma and termed the process clasmatosis.

The movement of melanin granules in response to light was followed in several melanocytes for a period of 15 minutes. When first observed, the pigment granules were uniformly distributed in the cells. Gradually the granules migrated to the peripheries. Tightly packed knobs of melanin formed along the borders of the cells, while the cell bodies became clearer and free of pigment. The process was reversed when the light from the microscope lamp was cut down.

Large melanocytes, with dendrites withdrawn, were also present in many cultures. They were particularly prevalent in the tumor of a striped-sided hybrid. The cells assumed an irregular, plaquelike shape and had evenly distributed melanin granules (FIGURE 5). They appeared to be mature cells and had some of the characteristics of melanophores. Similar cells were called juvenile melanophores by Marcus and Gordon.[40]

Small, spherical melanocytes were frequently present in actively growing cultures (FIGURE 6). The dendrites were withdrawn as the cells

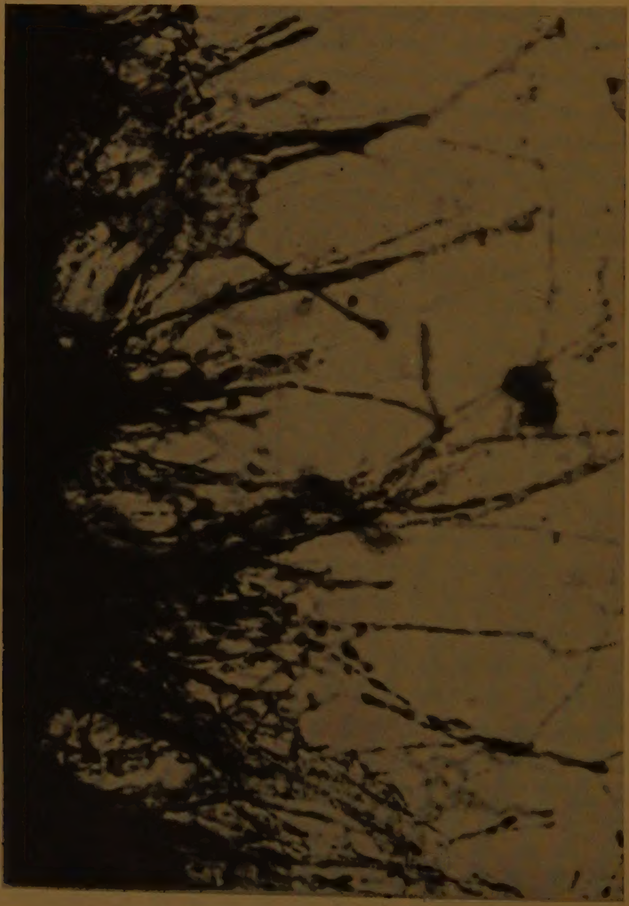
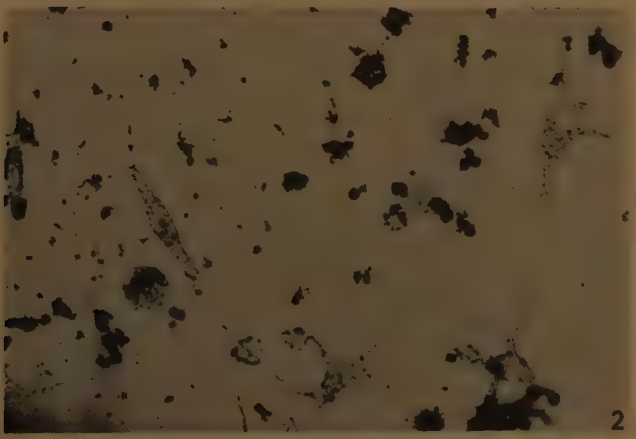


FIGURE 1. Dendritic processes of melanocytes extending from the margin of an explant of a living 1-day melanoma culture from an *Sb* hybrid. ×540.

became free of the explant. The spherical shape may be brought about by several processes: (1) mitosis; (2) anaplasia; and (3) the liquefaction of the culture medium. All these features are characteristic of malignant cells in tissue culture. The greater the number of rounded cells, the more liquid was the clot.

Actively growing cultures of advanced tumors also produced dendritic, round, and irregularly shaped cells with a granular cytoplasm, but no pigment (FIGURES 7 and 8). These cells appeared to be young melanocytes that had not synthesized visible pigment. The only cells that contained melanin in these active cultures were the outwandering melanophores and the phagocytic macrophages. The colorless, almost trans-



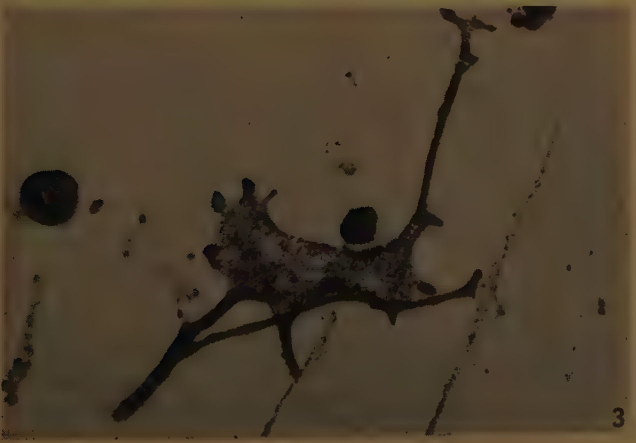


FIGURE 2. Living melanocytes from melanoma culture of an *Sb* hybrid. ×570.

FIGURE 3. Large melanocyte, with coarse, black granules and heavy dendrites, from melanoma culture of *Sr* hybrid. ×570.

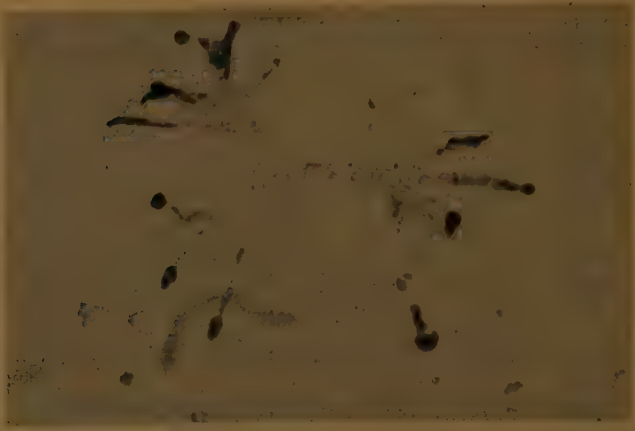


FIGURE 4. Living melanocyte with clumps of melanin at tips of the dendrites, in a culture from an *Sb* hybrid. ×570.

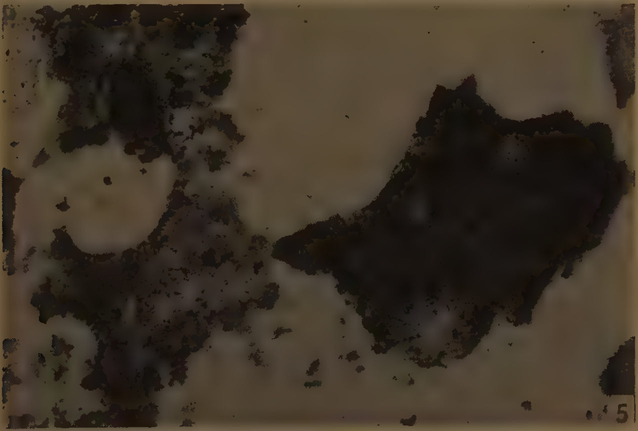
parent areas that frequently develop in advanced black tumors *in vivo* may be attributed to the active proliferation and accumulation of these immature pigment cells.

Melanophores. The large, melanin-carrying cells that impart the black color to the overgrowths are the macromelanophores. Variations in the degree of blackness of different tumors, or different parts of the same tumor, are due to the number of macromelanophores present. In late stages of the melanomas, macromelanophores disintegrate, leaving the tumorous tissue mostly gray or white. At the same time, the melanoblasts and melanocytes become the preponderant cells.[41]

Within the explant, the contained pigment of the melanophores was usually dispersed and the cells appeared stellate. Occasionally, the melanophores wandered into the outgrowth zone and assumed an ameboid shape (FIGURE 9). They moved slowly and, in 1 to 2 weeks, they became free of the explant. Melanophore-pigment granules were coarser and blacker than those in melanocytes. Frequently the cells were so solidly packed with pigment that individual granules were obscured and the cells appeared homogeneously black. Most of the melanophores were multinucleate. It is possible that these pigment cells actually represent the final, completely differentiated stage of melanocyte development. There was never any suggestion of cell division in melanophores, and they could not be cultivated *in vitro*. When melanophores were destroyed in the explant, they were never replaced. Those melanophores that migrated into the culture medium usually contained vacuoles, indicative of a degenerating process.

Macrophages. The macrophages appeared in the outgrowth zone soon after explantation, and they remained an abundant cell type throughout the life of the cultures. Most macrophages assumed a round shape and were laden with clumps of brown and black pigment in addition to other cellular debris. Sometimes they were so solidly engorged with melanin that they resembled inert black spheres. Giant-sized macrophages were frequently formed by the fusion of smaller ones (FIGURE 10).

The macrophages were motile in tissue culture, and moved by means of small, cilialike projections, or by a thin, undulatinglike membrane. The tapering projections were transitory, for they were rapidly extended and retracted. Frequently there was a flow of cytoplasm into a small projection, and it became branched to produce a tiny dendrite; finally, it



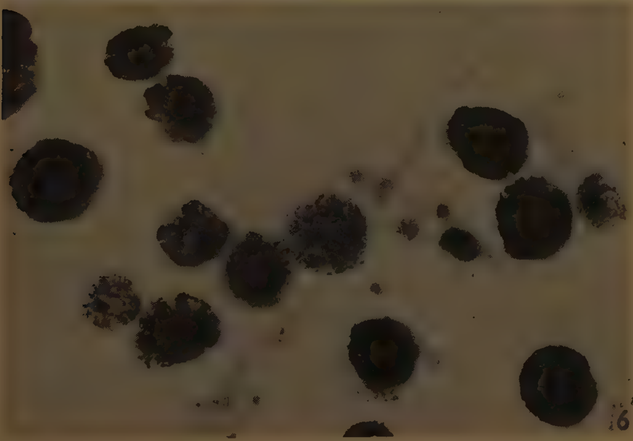


FIGURE 5. Plaquelike melanocytes in a tumor culture from an *Sr* hybrid. ×1330.

FIGURE 6. Spherical melanocytes from a melanoma culture of an *Sb* hybrid. ×1330.

broadened to form a pseudopodium. Momentarily, it reverted into a fine, ciliallike process or it lost its identity within the cell cytoplasm. Holtfreter[42] described a similar corona of minute pseudopodia in cultures of amphibian cells that were in the process of flattening out onto the glass. It is possible that these processes are locomotor in function, and they may be instrumental in the selection of migratory pathways.[43]

Fibrocytes. The main mass of the tumor is supported by mesenchymal connective-tissue cells that appeared on the first to third days in culture. They are spindle-shaped cells with 1 to 2 oval nuclei, each containing 1

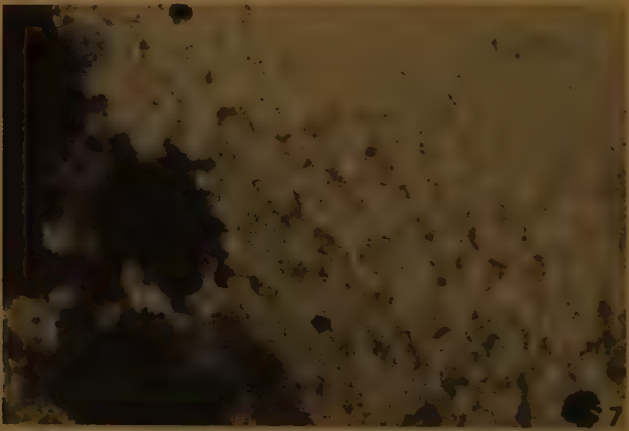
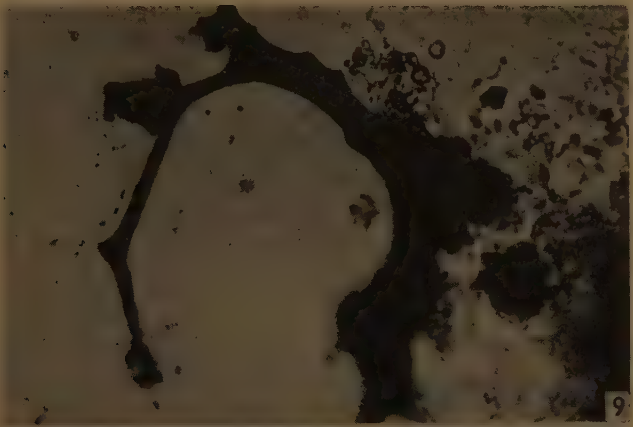


FIGURE 7. Pigmentless, granular cells in an actively proliferating melanoma culture from an *Sb* hybrid. ×570.

FIGURE 8. Living dendritic cells with colorless cytoplasmic granules in a melanoma culture from an *Sb* hybrid. Note the various shapes, from round to highly dendritic, assumed by these cells. ×570.

or 2 nucleoli, and they are known as fibrocytes. Anastomosing sheets of spindle cells were formed in cultures containing a rich fibrocyte growth. Some of the fibrocytes contained granules of melanin that were scattered around the nucleus. Fibrocyte melanin is believed to be of exogeneous origin.[44]

Epithelial cells. Hyperplasia of the epithelium, overlying the melanoma, is associated with the diffuse contact of the tumor cells with the epithelium. When cultured *in vitro,* the epithelial cells proliferated in extensive, flat sheets on the first day (FIGURE 11). Frequently the sheets were divided into sections by septa or cordlike processes, or they were



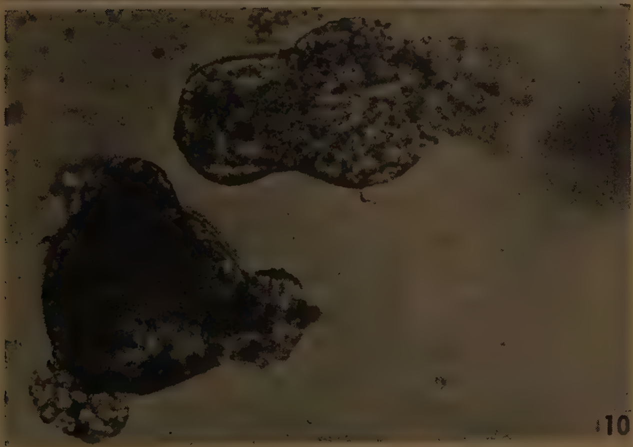


FIGURE 9. Living melanophore that migrated from explant into outgrowth zone during a period of 12 days. ×540.

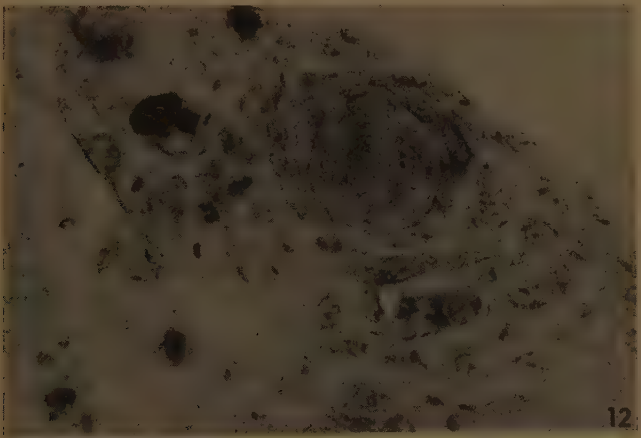
FIGURE 10. Living macrophages fusing to form aggregates. ×570.

FIGURE 11. Flat epithelial sheet in a living culture. ×120.

organized into pearl-like structures (FIGURE 12). Occasionally the epithelial cells failed to form sheets; instead, they formed tongues of closely clustered cells. The mass appeared to be thrust out of the explant (FIGURE 13). The border of the tongue was always a definite, well-formed membrane that sometimes contained long, fine, hairlike cilia that beat with a vigorous sweeping motion. This may have been the result of tension exerted on the cytoplasm which adhered to the coverglass or to the medium.

The epithelial cells were polyhedral in shape with 1 or 2 spherical nuclei, each with 1 or 2 round or oval nucleoli. The cytoplasm contained numerous inclusions in varying sizes that were spheroidal, refractile, and greenish in color (FIGURE 14). Some of the larger bodies were irregularly polyhedral in shape: an indication that they may be actual bodies rather than vacuoles. A few of the globular bodies had hollow centers. Frequently many of these inclusions fused together to form large, intracellular masses (FIGURE 15).

In totally black tumors, the epithelial cells that emerged from the explant had black-pigmented globules in addition to the usual greenish, refractile ones. Both types of inclusions were present in the same cell (FIGURE 16). In lighter-colored tumors, black epithelial globules were not observed until the start of the second week *in vitro*. On careful focusing, the pigment appeared to be applied to the periphery of the spheroids, as the centers remained refractile. When tumors synthesized brown



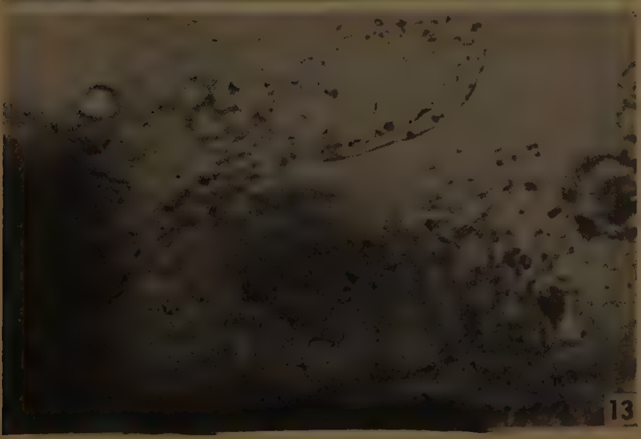
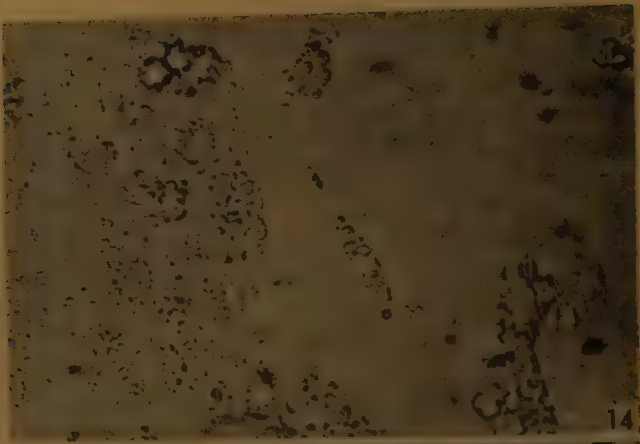


FIGURE 12. Pearl-like formation in an epithelial sheet from a melanoma culture. ×1330.

FIGURE 13. Margin of a living culture with 2 tonguelike masses of small, round epithelial cells. ×540.



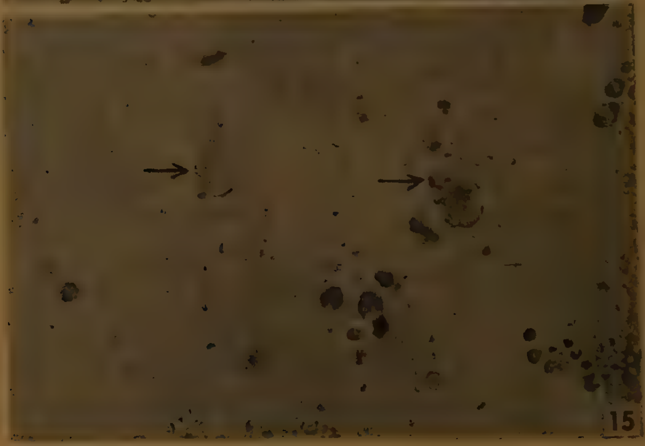


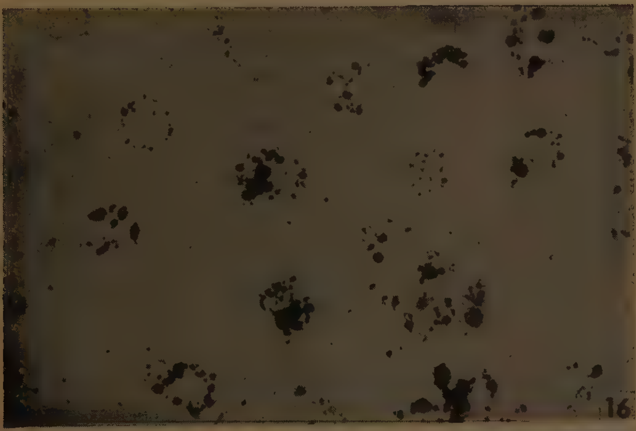
FIGURE 14. Living epithelial cells with numerous refractile inclusions in the cytoplasm. ×570.

FIGURE 15. Epithelial cells with masses of fused globules. ×1330.

melanin, as in some spotted-belly *(Sb)* hybrids, the epithelial globules were coated with brown granules.

Within an epithelial sheet an area was often present where the cells were devoid of refractile globules. Instead, the cytoplasm appeared to be sculptured into delicate, concentric rings or ridges that resembled the pattern of a fish scale. The core of the rings had no consistent relationship to the nucleus (FIGURE 17). The rings were similar in appearance to the lamellae, present in the ultrastructure of pancreas, liver, intestinal epithelium, and tumor cells, as seen with electron micrographs.[45-47]

Comparison of growth characteristics in tissue culture. Different fragments of the same tumor exhibited variations in growth rate and in cell types that developed. Rarely did a single culture contain all the cell types involved in the tumor mass. TABLE 1 lists the frequency with which 4 cell types appeared in the outgrowth zone of cultures from the melanomas studied. It can be seen from the table that the melanocyte was the prominent cell type in cultures of fish melanomas. Melanophores, although numerous in the explant, rarely appeared in the outgrowth zone. The number of actively proliferating melanocytes was greater in ventral tumors from *Sb* hybrids than in dorsal fin and flank overgrowths from *Sd* and *Sr* hybrids. In addition, melanocytes from the latter 2 tumors grew very slowly, often requiring as much as 6 to 9 days before they became



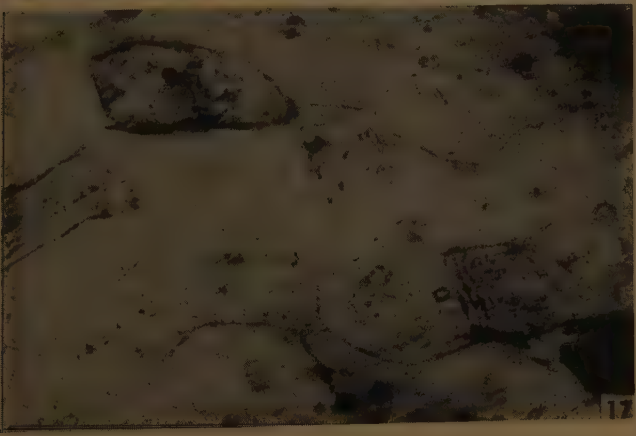


FIGURE 16. Living epithelial cells with both black and greenish refractile inclusions. ×570.

FIGURE 17. Epithelial cells with cytoplasmic rings. Note the absence of globular inclusions. ×1330.

TABLE 1

PERCENTAGE OF CULTURES EXHIBITING 4 CELL TYPES FOUND IN MELANOMAS

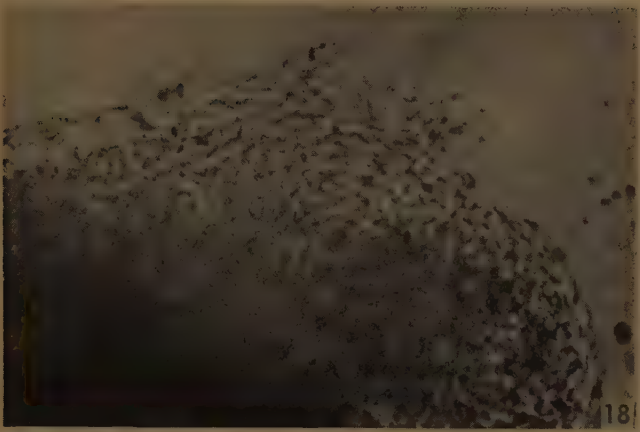
Tumor and pedigree of hybrid	*Melanocytes*	*Epithelial sheets*	*Melanophores*	*Fibrocytes*
Sb 354, spotted belly	49%	30%	8%	9%
Sb (354)1	34	18	13	27
Sb (354)2				
soft tumor	57	20	6.3	
fibrous tumor	4.7	0	0	31
Sb (354)3				
soft tumor	61	61	11	28
fibrous tumor	42	50	4.2	25
Sb (354)4				
soft tumor	57	40	3.3	6.3
fibrous tumor		growth very scant		
Sd 444, spotted dorsal	30	0	20	20
Sd 316	11	69	0	11
Sr 354, stripe-sided	23	15	6.2	2

evident. Melanocytes, induced by the *Sb* gene and modifiers, usually appeared on the first day of culture. Melanocytes that were manifestations of the *Sr* and associated factors were mostly mature, large cells with heavy, black pigment granules. In contrast, melanocytes from tumors of *Sb* hybrids were mostly small cells with fine, powderlike granules that were colorless or brown.

A large percentage of cultures produced epithelial cells that grew extensively and rapidly. Epithelial hyperplasia, in association with the pigment tumors, was reported by Levine[3] from histological sections. Levine described a tumor from an *Sb* hybrid in which tongues of epithelial cells grew downward and infiltrated the growth to the extent where clusters of tumor cells were isolated in the corium. Epidermal growth from tumors initiated by different pigment genes was mostly similar *in vitro*.

Two ventral tumors referable to the *Sb* gene and modifiers were frequently present in the same animal. The masses had different textures. One was usually hard and fibrous and exhibited feeble growth *in vitro*. The other was soft and grew vigorously. The cells that developed from the fibrous, opaque, white nodules were small spindles with small nuclei and extremely short processes. They adhered closely to one another and formed dense clusters. Frequently a spindle capsule formed around the periphery of a cluster, thus restricting the outgrowth of the cells (FIGURE 18). Few loose cells were ever present in these cultures. Such nodules within the tumor might be thought of as scar tissue.

The replacement of the entire core of the tumor by fibrous scar tissue occurred in the dorsal fin melanoma of the *Sd* 316 hybrid. On gross examination, the tumor was lobulated and totally black. When dissected, the



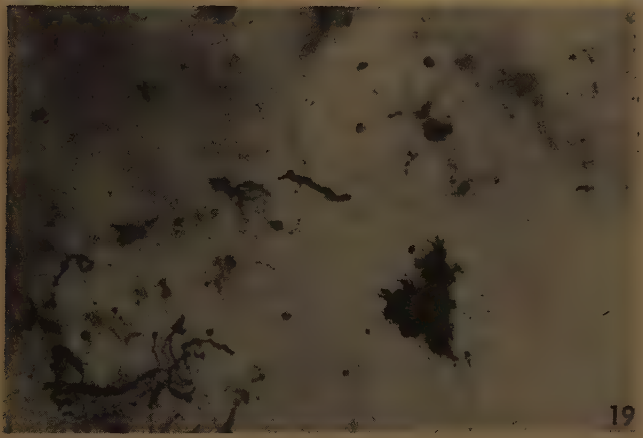


FIGURE 18. Living culture from fibrous white tumor tissue. Note the limiting capsule that restricts the outgrowth of the cells. ×540.

FIGURE 19. White explant containing scattered, fragmenting melanophores. ×130.

inner mass of the tumor was found to be white and opaque. The black pigment was limited to a thin surface layer. The tissue, probably necrotic, had an odor of putrescence. Microscopic examination of the white tissue revealed a few scattered, fragmenting melanophores (FIGURE 19). Growth, *in vitro,* from this light area of the tumor was sparse.

Inclusions that are not normal constituents of the proliferating cells were frequently present. Erythrocytes were identified in epithelial cells and in melanocytes (FIGURE 20). Phagocytosis occurred in those epithelial cells that had become separated from the sheet or aggregate. Loustalot *et al.*[48] likewise observed phagocytosis of whole cells in sections of the Cloudman S91 melanoma, but they were not certain whether the phagocytic cells were tumor cells or macrophages.

Cytological Properties

In addition to hematoxylin and eosin, the Feulgen reaction, May-Grunwald-Giemsa stain, and methyl green-pyronin proved extremely useful in the morphological analysis.

All shades of pigment granules in melanocytes, from light brown to black, proved to be refractory to stains. Frequently the granules were packed so tightly that the nuclei were obscured. The number of nuclei appeared to be a function of cell size, indicating a failure of cytokinesis. That this is not a peculiarity of melanocytes *in vitro* is indicated by the multinucleate cells observed by Levine[3] in histological sections of these tumors. It is believed that amitotic division occurs in the cells of fish melanomas.[3, 49, 50] The nuclear-cytoplasmic ratio was not unusual, and the nuclei were not deformed. The cytoplasm stained poorly with eosin, and often exhibited definite basophilia, staining pale blue.

Intensely basophilic bodies were occasionally present in the cytoplasm of melanocytes, fibrocytes, and epithelial cells. Observations on many cells indicated the nuclear origin of these bodies from extruded chromatin clumps and from constricted nuclear fragments. This was confirmed with both the methyl green-pyronin stain and the Feulgen nucleal reaction. The spherical bodies proved to be intensely positive for DNA, as they stained dark purple with Feulgen's technique and dark green with methyl green. Feulgen-positive bodies, in the cytoplasm of certain plant cells, were observed by Sparrow and Hammond,[51] who suggested that they

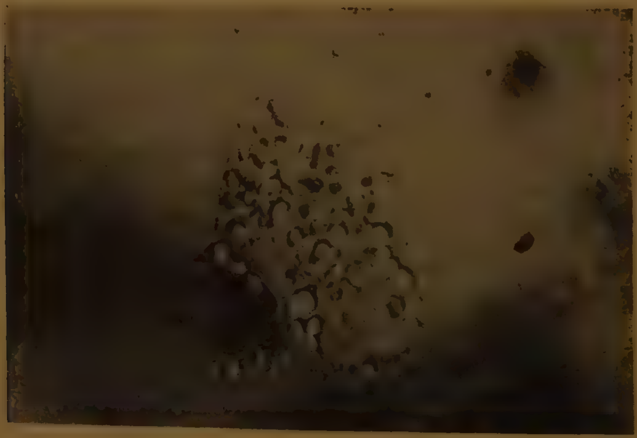
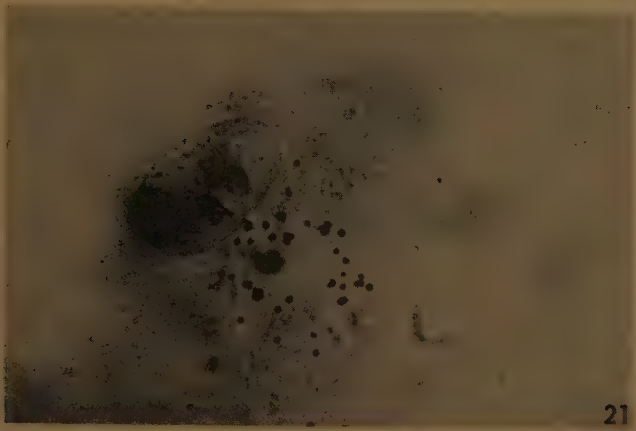


FIGURE 20. Epithelial cells with engulfed erythrocytes. ×1000.



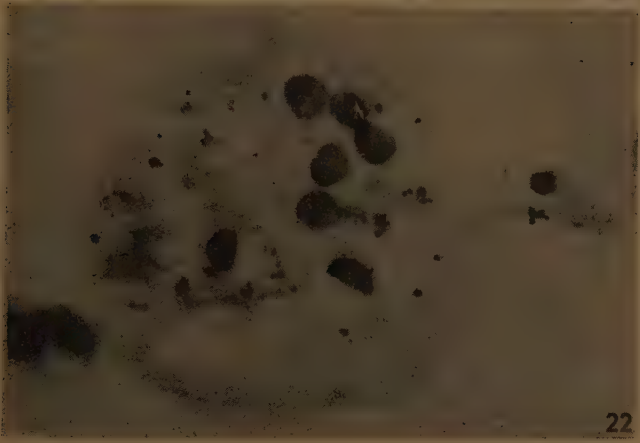


FIGURE 21. Nucleus containing 2 nucleoli. One is round with vacuole in center, and the other is branched. Note the melanin granules in the cytoplasm. ×1330.

FIGURE 22. Multinucleated cell from a melanoma culture. Hematoxylin and eosin stain. ×1330.

may function as a site of protein synthesis, or they may be converted to RNA in the cytoplasm.

The greenish, refractile globules in the epithelial cells showed variable staining with eosin, ranging from red to pale pink, while some globules remained unstained. The faint rings or lamellae identified in some of the living epithelial cells did not stain with hematoxylin and eosin.

The numerous cytoplasmic inclusions of the epithelial cells frequently caused deformations of the nuclei, presumably due to pressure.

Both direct nuclear division and mitotic division were observed in these cells. The cytoplasm was basophilic.

Large, epithelial-like cells, with bizarre-shaped nuclei and nucleoli and with much diffuse filmy cytoplasm were present in many cultures. Frequently they were united into anastomosing, branching formations, with no limiting cell membranes. The nuclei measured 10 to 20μ. The nucleoli exhibited a variety of shapes, that is, round, ameboid, or complexly branched. Each nucleus had 1 to 3 nucleoli that usually did not resemble each other (FIGURE 21). Many of the large nucleoli stained pink with eosin except for the limiting margin, which stained with hematoxylin. Thorell[52] believes that large nucleoli, a characteristic of rapid growth, have an affinity for acid dyes, due to proteins rich in hexone bases that obscure the acid groups of nucleic acids. Scattered melanin granules were present in the diffuse cytoplasm.

Other large cells, 50 to 60μ in diameter, were frequently found in the tumor cultures. The nuclei numbered from 1 to 40 and were sometimes arranged in a horseshoe-type configuration, but more often they were scattered haphazardly throughout the cell. The presence of incompletely separated nuclei suggested that the multinucleate condition was the result of direct nuclear division without cytoplasmic division (FIGURE 22). Ludford and Smiles[53] concluded that the tendency to form giant cells appears to be a universal attribute of malignant growths. In tissue culture, however, the significance of such cells is not clear. The conditions of growth may be such that the delicate mechanism of cytokinesis is upset, without disturbing karyokinesis. A controlled medium would be necessary to rule out the numerous variables inherent in the artificial environment.

Cytochemical Properties

Nucleic acids. The methyl green-pyronin stain revealed the general location of the 2 types of nucleic acid. The nuclei were blue-green, characteristic of DNA. There was little variation in the intensity of the green, except in cells with fragmenting nuclei. The clumped chromatin was an intense green. The DNA was frequently arranged in the form of a chain of green beads surrounding the nucleolus. Ludford[54] observed peripheral aggregations of nucleolar chromatin in mammalian cells, and he considered this to be part of the nucleolar system.

Pyronin staining occurred in the cytoplasm and nucleoli. It is thought to be an indicator of RNA as well as other constituents.[11] Nucleoli stained darker than cytoplasm. When pyronin B was used singly, without methyl green, the nuclear periphery stained pink in addition to the nucleolus and cytoplasm. Frequently the pink periphery was composed of a beadlike chain. Caspersson and Schultz,[55] using ultraviolet absorption methods, found the maximum cytoplasmic concentration of RNA to be close to the nuclear membrane. When pyronin-stained cultures were restained with the methyl green-pyronin mixture, the pink nuclear peripheries were obliterated. Instead, a heavy green deposit was present at this site. The nucleoli and cytoplasm were unchanged. A heavy green layer surrounded the nucleoli, while the remaining nuclear area was a lighter green. From the results of these single- and double-staining techniques, there appears to be an interaction between nucleolar RNA and nuclear DNA at the margin of contact between nucleolus and karyolymph, and that the karyotheca is also a site of RNA and DNA activity.

A rough quantitative estimate of DNA was made by extraction with trichloracetic acid for different lengths of time, followed by staining with methyl green-pyronin. No staining occurred after boiling for 1 to 1½ minutes in trichloracetic acid at 90° C. This timing was compared with that necessary to produce the same results in amelanotic melanoma cells.

The distribution of DNA was confirmed by the Feulgen reaction. A positive reaction was present in the nuclei, while the nucleoli and cytoplasm remained colorless. The latter were made visible with a fast green counterstain. Pigmentless melanocytes were frequently observed in which the DNA was arranged in the form of purple beads at the periphery of the nucleolus and at the nuclear membrane. This coincides with the distribution obtained with methyl green-pyronin.

The May-Grunwald-Giemsa technique gave additional information concerning nucleic acids. The chromatin stained red-purple, which is indicative of DNA. The RNA of the cytoplasm and nucleoli stained blue. Dark, purplish-blue droplets that resembled the hematoxylin-positive, Feulgen-positive, and methyl green-positive inclusions, were present in the cytoplasm of many cells. This dark staining with the May-Grunwald-Giemsa dyes is believed to be due to a combination of both DNA and RNA.[14] RNA may therefore be assumed to be present, in addition to the already demonstrated DNA, in the pinched-off nuclear fragments. Small pink granules were occasionally observed in the cytoplasm of the epithelial cells.

Refractile bodies. The refractile globules of the epithelial sheet cells were mostly extracted following differentiation of the methyl green-pyronin mixture and dehydration with tertiary butyl alcohol. Occasionally a few globules resisted extraction and became stained with pyronin. The staining was not uniform, and it ranged from a barely visible pale pink to a dark pink in the larger globules. Dark green spherical bodies were scattered throughout the epithelial sheets, which were identified as pyknotic nuclear fragments. Methyl green, used as a single stain, did not color the refractile globules. When pyronin was used alone, the globules were stained red. In neither case was tertiary butyl alcohol used as a dehydrating agent, and the globules were not extracted.

Feulgen reaction. The refractile globules reacted intensely, but not consistently, to the Feulgen technique. When positive, the globules recolorized the leukofuchsin reagent to a dark, red-purple. This was in contrast to the lighter purple stain of the nuclei (FIGURE 23). Not all the globules within a single cell became stained and, frequently, the cells of an entire sheet failed to react, whereas another sheet, which proliferated from the same explant, gave a positive reaction. Whole cultures were frequently completely negative, although there was no variation in the Feulgen hydrolysis or Schiff's reagent used. Apparently a difference in chemical composition of the globules exists, which may be attributed to one of two factors: (1) two chemically different inclusions are present, one of which sometimes predominates; (2) the positive and negative reactions to the Feulgen technique may represent different stages in the formation of one type of secretion droplet. The age of the culture was not a factor in yielding positive or negative Feulgen globules.

Variations in the extent of staining within the individual droplets were also evident. Frequently the rim of the globule was dark purple and the center remained colorless, or the dark purple of the rim became progressively lighter toward the center of the globule, leaving an eccentric inner, unstained portion. Less frequently, solid purple spheres were present. In a few positive globules, the color was not the usual dark red-purple, but a pale lavender. Such a staining always occurred when melanin was present in the epithelial cells. When a fast green counterstain was employed, the nucleoli stained green, but the Feulgen-positive rings

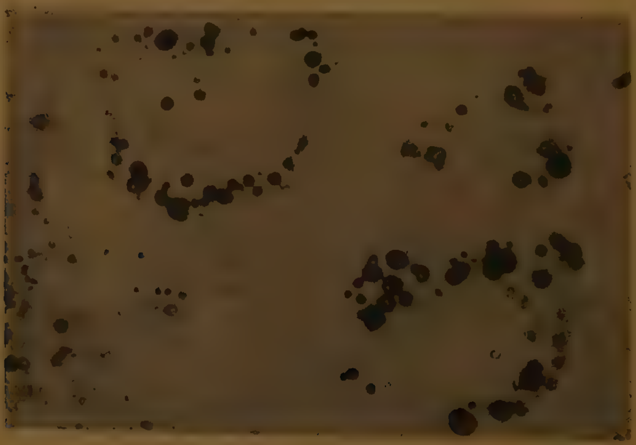


FIGURE 23. Feulgen-positive inclusions in epithelial cells. ×1330.

maintained their colorless centers. Occasionally, Feulgen-negative globules were stained pale green.

The partial staining of the globules with the Feulgen reaction is in contrast to the complete globule staining with eosin and pyronin. In addition, the staining with the latter dyes was always faint, whereas the positive Feulgen staining was usually intense.

Polysaccharides. The periodic acid-Schiff reagent (PAS) was used for the detection of 1,2 glycol linkages in an attempt to identify the refractile globules of the epithelial sheet cells. A positive reaction is ordinarily obtained by this method with mucopolysaccharides, glycogen, and unsaturated lipids. Practically all the refractile globules remained unstained, but occasionally 1 or 2 dark purple or pink globules were found in a sheet.

A metachromatic reaction was not obtained with toluidine blue in the epithelial cell globules. Acid mucopolysaccharides, which are esters of sulfuric acid, give γ (red) metachromasia. Instead, a few scattered globules were orthochromatic, and they stained blue. Most globules, however, were refractory to the dye. Partially stained globules, containing 1 to 4 blue circular patches, were present. Pyknotic nuclear fragments were a dark, inky blue.

When mucicarmine was used for the staining of mucin, approximately one half of the epithelial-cell globules stained dark pink. According to Pearse,[17] the basis of this reaction is merely the basophilia of an acid substance, and consequently it has been used to stain acid mucopolysaccharides. In itself, however, the mucicarmine stain cannot be considered specific for mucin. From this it may be deduced that the unstained globules were not basophilic. Rose bengal, also a mucin indicator in the category of mucicarmine, stained most of the globules. A characteristic intensity of color was not obtained but, instead, the globules varied from bright red to pale pink. Both unstained and stained globules were observed in the same cell. The alcian blue test, specific for acid mucopolysaccharides, was completely negative. No reaction was produced in the globules with the ammoniacal silver technique, which depends on the formation of silver complexes with aldehydes of mucin and glycogen.

Lipids. The Feulgen-positive globules were studied by means of the plasmal and pseudoplasmal reactions, since the plasmalogens react similarly to DNA when treated with leukofuchsin. Extensive studies of the plasmal reaction have established it as a reaction of acetal phosphatides whose aldehyde groups are made reactive by mild hydrolysis or oxidation.[22-26] The refractile bodies of the epithelial cells did not react to the plasmal or pseudoplasmal techniques.

Since unsaturated fatty acids may be oxidized to form hydroperoxide bonds (-OOH), that may subsequently be opened to form keto and hydroxy groups that finally form aldehydes on breaking of the carbon chain, a positive reaction with Schiff's reagent may be demonstrated. Various lipid tests were therefore performed to determine if the Feulgen-positive globules of the epithelial cells contained unsaturated fatty acids that may have been oxidized during fixation. No staining was obtained with Sudan IV or Sudan black B with the usual methods for these dyes. When, however, the Sudan black B technique was carried out at 60° C. for 16 hours, small areas within several large globules stained light blue, but again the majority of globules remained unstained. In order to determine if the specific cultures that proved refractory to the fat stains would also be refractory to the Feulgen stain, the same cultures were subjected to the nucleal reaction after being stained with the Sudan dyes. The lipid-negative globules recolored the leukofuchsin after hydrolysis.

Nile blue A stained a few globules pink, whereas the majority of globules were a bluish-purple. The nuclei stained blue. The same globules that gave a negative reaction with Sudan IV, were positive on restaining with Nile blue A. The specificity of the Nile blue A stain for lipids is questioned by Lillie.[56] It is believed that all basophilic substances are stained in varying intensities.

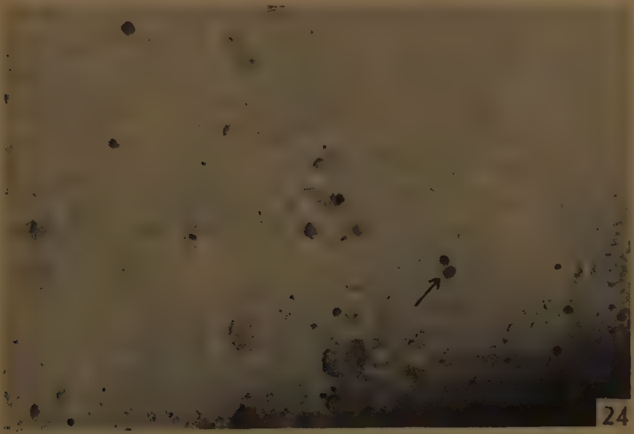
Menschik's[27] Nile blue method for phospholipids left the majority of globules unstained and in their original greenish, refractile state. An occasional definitely blue globule was present, however, as well as a few partially stained globules (FIGURE 24). The nuclei of the epithelial cells stained pale blue, while the nucleoli and nuclear membranes were a darker blue. Baker's[28] acid hematin test for phospholipids was also essentially negative, with just a few blue-stained globules.

In order to explore the possibility that the globules were a lipoprotein complex, the epithelial sheets were hydrolyzed to unmask the lipid and then stained with Sudan black B.[29] The majority of globules remained unstained, but a few were colored blue or partially blue in spots. The outer rims of some stained blue. Examination of the refractile globules by polarized light did not reveal the birefringence characteristic of crystalline lipids.

Osmium tetroxide was reduced by some of the globules after a long-term exposure of 5 days. Both black and colorless globules were always present in the same cell (FIGURE 25). No definite pattern as to proximity of the osmicated granules to the nucleus could be established. Globule size was also no criterion for reactivity, as both large and small globules were equally positive and negative. Careful focusing on the positive globules indicated that the reduced osmic acid was sometimes present only on the surface. The interior of the bodies retained their greenish, refractile quality. Overnight incubation in osmic acid had no effect on any of the globules, which is an indication of the absence of unsaturated lipids.[57]

Porter's[58] method of osmic-acid fixation designed to show the submicroscopic basophilic lamellae was applied to epithelial sheets that contained cells with delicate concentric rings. Osmic acid did not increase the density nor stain the ring structures.

Extraction techniques. Dehydration of the epithelial sheets in graded alcohols resulted in a loss of the refractile property of the globules and a decrease in their size. When the same cells were hydrated again, the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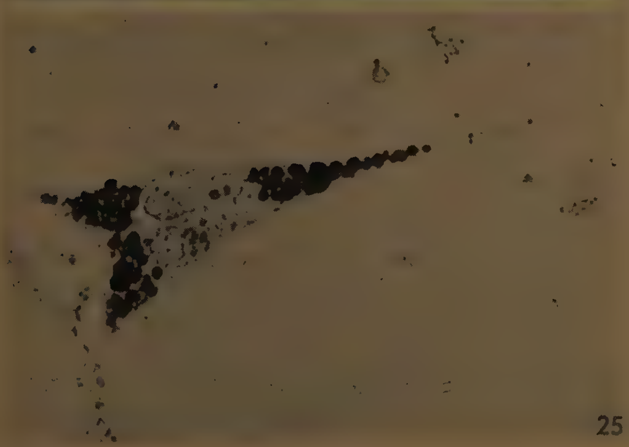
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FIGURE 24. Phospholipid-positive patches within an epithelial-cell globule. ×1330.

FIGURE 25. Blackened globules in an epithelial cell treated with osmium tetroxide. ×1330.

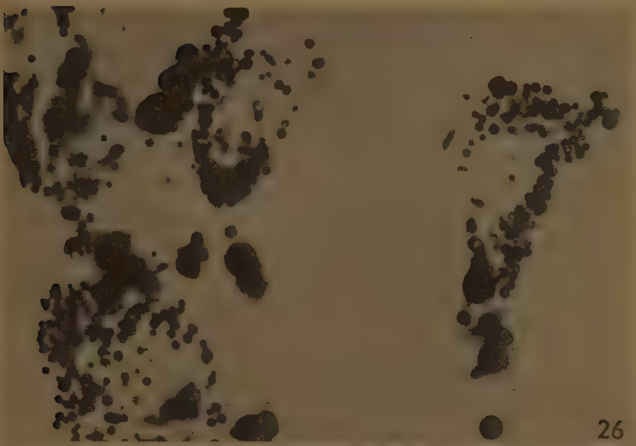
globules were restored to their original size. The globules resisted extraction with acetone, xylol, and ethyl alcohol. Pyridine extraction at 60° C. for 24 hours left most of the globules intact. A few of the smaller ones, however, were removed. Extraction with chloroform and methanol for 24 hours at 60° C. had little effect, except to remove the refractile quality of the globules. They acquired a dull, greenish-yellow hue. Extraction was consistently obtained with tertiary butyl alcohol and absolute ethanol, 3:1.

Performic acid and Schiff reaction (PFAS). A brilliant purple-red color was developed in the majority of refractile globules with the performic acid-Schiff technique (FIGURE 26). As with the nucleal reaction, the staining was not uniform. The outer rims were strongly stained, while the centers were a lighter pink or colorless. A few solidly stained, purple globules, as well as completely colorless ones, were also present. The globules did not respond as consistently, and in such great numbers, to any other reaction as to the PFAS. When melanin granules were associated with the globules, no staining occurred. Melanin-free globules in the same cell were positive, but the intensity of the color was diminished to a pale lavender. Melanin had the same effect on the Feulgen staining of globules. No other cellular components responded to the PFAS reaction. A side effect, however, was the bleaching of black melanin by the performic acid oxidation to a brown color. Lillie[59] observed a similar reaction in ocular melanin, which became bleached during the PFAS technique. The concentric, lamellar, epithelial lines remained unstained.

Negative reactions. In the identification of the refractile globules, negative reactions were obtained for the following substances, using the recognized techniques for their demonstration: (1) amyloid – methylene violet and Congo red methods;[56] (2) vitamin A – Carr-Price reagent;[56,60,61] (3) ceroid – Verhoeff's acid-fast carbol fuchsin test;[62-64] (4) lipofuscins – chrome alum hematoxylin method;[17] (5) ascorbic acid – acid silver nitrate technique;[17] (6) calcium – alizarin red method,[17] extraction with citrate buffer, and chelation with disodium versenate.

Glycogen. There was a reaction in both the pigment cells and epithelial cells to treatment with periodic acid and Schiff's reagent. Small, PAS-positive granules were distributed in the cytoplasm. The granules were frequently displaced to the periphery of the cell (FIGURE 27), a common phenomenon caused by diffusion currents initiated during the fixation process.[65] Some of the granules stained dark purple, while others were a lighter pink. Salivary digestion, before the PAS treatment, removed the purple granules, indicating the presence of glycogen. The pink-stained granules were saliva-fast.

Lipids. A positive plasmal reaction was found in the lightly pig-



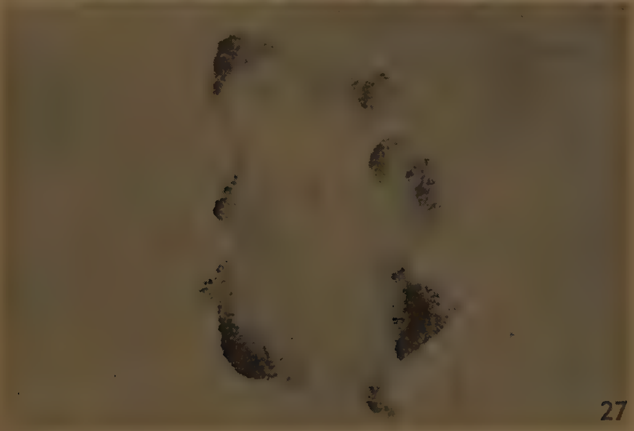


FIGURE 26. Brilliant purple globules in PFAS-treated epithelial cells. ×1330.

FIGURE 27. PAS-positive glycogen granules at periphery of epithelial cells. ×1330.

mented melanocytes and in the epithelial cells. Small pink granules became visible in the cytoplasm. The pseudoplasmal technique revealed fainter pink granules in the cytoplasm of a few epithelial cells. Pseudoplasmal was not detected in the pigment cells. Control tests for free aldehyde, which might also cause positive results, were completely negative.

In old cultures, a few Sudan IV-stained inclusions were observed in a variety of cells. This is probably the result of fatty degeneration, a common occurrence in old tissue cultures.

Thiol groups. Epithelial cells were consistently positive for thiol groups by both the ferricyanide and azo dye methods. Melanocytes did not give the thiol reaction. The epithelial-cell nuclei and nucleoli were blue with the ferricyanide reaction. The cytoplasm surrounding the nuclei was blue, and the color gradually faded as the distance from the nucleus increased. Pyknotic nuclear fragments were dark blue. Nuclear thiol can frequently be demonstrated in actively metabolizing cells. Thus the thiol content of nuclei was found to increase during the period of active growth in the embryonic life of grasshoppers.[66] Yao[67] demonstrated nuclear thiol in the *Drosophila* oöcyte.

The reaction of the epithelial globules was variable. In some sheets the majority of globules stained blue. In others, there was no reduction of ferricyanide, and the globules were unstained. Control cultures for the ferricyanide reaction were pretreated with $HgCl_2$. Only partial inhibition was obtained, however. When iodoacetic acid was used as an inhibitor, inhibition was complete at times, but it could not be obtained consistently.

The more specific technique of Barnett and Seligman[35] for protein-bound thiol groups gave essentially similar results as the ferricyanide method. The epithelial cell nuclei and the cytoplasm were pale pink, indicating few or widely separated thiol groups. The nucleoli were bluish-pink, indicating a greater concentration of these groups. Melanocytes did not stain. Complete inhibition of the reaction was obtained by pretreatment with iodoacetic acid.

Alkaline phosphatase. The nuclei of melanocytes were clearly defined by the Gomori technique and were intensely positive. The nuclear membranes and nucleolar rims were coal-black. The chromatin was black, but with not as heavy a precipitate. The cytoplasm was light gray. Controls incubated without the glycerophosphate substrate were negative. The nuclei of epithelial cells also exhibited alkaline phosphatase activity, but the reaction was much less intense than in the pigment cells. Some of the epithelial globules were uniformly black, but most had a surface layer of individual black granules. In controls incubated without glycerophosphate the nuclei were unstained, but there were brown granules that adhered to the surface of the globules.

Melanin reactions. The colorless cytoplasmic granules of incompletely differentiated melanocytes reduced ammoniacal silver nitrate solutions, indicating a positive argentaffin reaction. The quantity of metallic silver formed varied with different cells. In some cells the granules were all black. Others had reddish-brown granules, while most cells had a mixture of reddish-brown and black granules. Explants that were a light tan color in the living state generally became coal-black after incubation in the silver solution.

Many of the refractile globules of the epithelial sheet cells were effective in reducing the silver solution. Smooth, coal-black, homogeneous spheres were formed, although colorless globules were also present in the same cells along with the strong reducers. There was no distinction between the active and inactive globules on the basis of size. On the surface of a few globules that were not completely black there were individual dark, reddish-brown granules. Globules ingested by fibrocytes and macrophages also formed metallic silver.

The argyrophil reaction, which involves impregnation with metallic silver, was demonstrated mainly by the pigment cells. The metallic silver is produced by an extraneous reducer added to the silver solution. Argyrophil substances are thought to be precursors of argentaffin. Masson[68] observed that the premelanin granules of human pigment cells were argentaffin-negative and argyrophil-positive. The colorless granules of the fish pigment cells became reddish-brown or black. The nucleoli and nuclear membranes stained black; nucleoplasm was brown (FIGURE 28). The majority of refractile epithelial globules remained unstained, but an occasional globule was coated with black or reddish-brown granules.

The acknowledged substrate for tyrosinase, 3,4-dihydroxyphenylalanine (dopa), was used to localize the melanogenic enzyme *in situ*. Tyrosinase activity was reported in the melanomas of xiphophorin hybrids by Fitzpatrick *et al.*,[69] who measured it manometrically. In the present studies, dopa oxidase activity was found in the pigmentless and pigmented melanocytes and in the epithelial cells. The nuclei and cytoplasm of the cells stained brown. The reaction was most intense in the nuclear membrane and nucleoli. The cytoplasmic staining was often darkest near

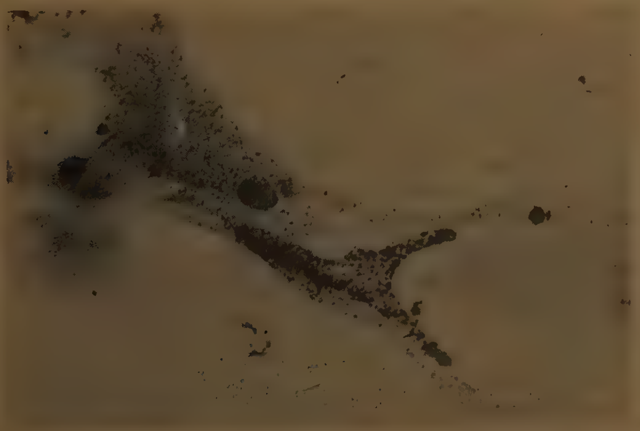


FIGURE 28. Positive argyrophil reaction in a colorless melanocyte. Some granules are stained black, others reddish-brown. The nucleolus and nuclear membrane are black. ×1330.

the nucleus, and it became less intense as the distance from the nucleus increased. The colorless granules of the pigment cells were colored brown with an intensity that often equaled that of the nucleoli. Some cells in the culture were always darker than others.

Some of the refractile epithelial globules were dopa-positive. A dark brown precipitate was formed on the surface of the globules that gave them a granular, irregular contour and obscured their refractile nature. Not infrequently, the positive reaction took the form of a dark brown homogeneous rim around the globules.

Control cultures kept in water at 100° C. for 15 minutes prior to dopa incubation failed to show enzyme activity. Likewise, inhibition was effected when the cultures were allowed to react with 1N HCl for 1 hour before exposure to the substrate. Controls incubated in buffer solution without the substrate showed no changes in the cells. Pretreatment with 0.1 per cent phenylthiourea for 1 hour failed to inhibit the dopa reaction in both the pigment cells and epithelial cells. Phenylthiourea is believed to be an inhibitor of the copper enzyme involved in melanin synthesis. In spite of this, strongly positive nuclei, nucleoli, cytoplasm, and refractile globules were obtained consistently. In fact, the positive reaction appeared to be more intense than in nontreated, dopa-stained cultures. A corresponding intensification of color, after treatment with phenylthiourea *in vivo*, was reported for amphibian embryos.[70] The depigmented embryos developed a more intense pigmentation than untreated controls when they were removed from the chemically treated water.

In the present experiments, phenylthiourea behaved as a competitive inhibitor, since it brought about inhibition only when present in the incubation medium along with the dopa substrate. The cytoplasm and epithelial globules were completely unstained, whereas the nuclei and nucleoli were light brown. Phenylthiourea has been shown to function in a similar manner *in vivo*.[71] When platyfish whose pigmented dorsal fins were excised were placed in water containing phenylthiourea, regeneration occurred, but pigmentation was delayed. The suppression of melanin synthesis was reversible, however, since the regenerated tissue became black after the fish were removed from the phenylthiourea-treated water.

The present studies indicate that sodium bisulfite produces a more complete inhibition of the dopa reaction than phenylthiourea *in vitro*. Inhibition was consistent and complete when sodium bisulfite was added to the incubation medium. On the other hand, when used as a noncompetitive inhibitor with pretreatment for 1 hour before the reaction, tyrosinase activity remained mostly positive.

When a buffered tyrosine solution was used as the substrate, no enzyme activity was observed. The addition of a ferric chloride catalyst[72] likewise elicited no reaction. With tyrosine plus a dopa catalyst,[73] however, a positive reaction developed in the pigmented and nonpigmented melanocytes and in the epithelial cells. The nuclei were brown. Nucleoli were a darker brown, while the cytoplasm was light brown or tan. Some of the refractile epithelial globules were covered with fine, brown granules. Others were smooth and brownish black. The reaction with tyrosine was not as intense as with dopa.

Once again, inhibition was not achieved with phenylthiourea when it was used for 1 hour prior to incubation in tyrosine plus catalyst. Instead, there was a more vigorous reaction in both the nuclei and cytoplasm of treated cells than in controls that were not pretreated. Complete inhibition of the reaction occurred when phenylthiourea was present simultaneously with the substrate. In a like manner, sodium bisulfite inhibited the reaction.

AMELANOTIC MELANOMAS

Gross Morphology

Melanomas in homozygous albino hybrids vary in color from pink to light gray to dark gray. The pinkish hue occurs in those tumors that contain no pigment, and it is due to the rich vascular supply. A caudal fin tumor, with 2 distinct sections, 1 gray and 1 white, was observed in a hybrid bearing the *N* gene and modifiers as well as the *ii* genes for albinism. Although both sections were attached and part of the same tumor mass, there was no blending of colors between the gray and white. The white area was distal to the pigmented and had replaced part of the caudal fin. This fragment became detached from the rest of the tumor and was sloughed off in the course of natural movements of the animal. The margin of the remaining tumor, which was in the fleshy caudal peduncle, was smooth, and it showed no signs of fraying or bleeding.

On dissecting amelanotic melanomas, the pigmented regions were usually found to be limited to the surface of the growth, while the interiors were mostly white. Hard, white nodules were frequently encountered within the tumor mass. All amelanotic growths were of firm consistency, so that evenly edged fragments could be cut for explanting.

Growth Pattern and Cell Types in Tissue Culture

The caudal peduncular tumors of albino hybrids, bearing the *N* gene, grew well in tissue culture. Growth was always accompanied by liquefaction of the plasma clot. Tumors of the dorsal fins from spotted dorsal *(Sd)* albino hybrids were slow-growing. The yield of cells was poor, and much cellular degeneration was apparent.

Microscopic examination of the explants revealed the presence of melanocytes and melanophores with variously colored pigment granules, that is, tan, light brown, dark brown, and black. A single explant had both black and brown cells, although there was usually a preponderance of 1 color type. Dendritic erythrophores with orange-red granules and xanthophores with canary-yellow granules were also prevalent within the tumor tissue. All amelanotic tumors examined contained pigmented cells, although some growths appeared pure white on gross inspection.

Most of the brown pigment cells had a roundish or irregular amorphous shape, and they contained evenly dispersed pigment granules. A few brown granular cells had the typical dendritic shape of melanocytes. Some of the dendrites were gnarled with swellings similar to the arms of the large melanocytes in typical melanomas. The brown cells migrated from the explant into the culture medium, but they did not divide.

Brown, melanophorelike cells in the explants resembled the melanophores of melanomas, except for the color of the granules. When black melanophores were present, they were punctate or in a state of disintegration. Intact, black melanophores were observed in an amelanotic melanoma of a newborn animal. After 2 weeks *in vitro,* the black cells disintegrated and all trace of them was removed. They were never replaced.

The cells that proliferated in tissue culture from the amelanotic melanomas included melanocytes, macrophages, fibrocytes, and epithelial cells.

Melanocytes. As in black melanomas, the dendritic, granular cells were the preponderant type in the culture medium. Dendrites appeared at the periphery of the fragment on the first day after explanting, and whole cells appeared 2 to 3 days later. The cells were extremely variable in shape and texture. Some were highly dendritic with delicate branched and forked processes (FIGURE 29). When the dendrites were withdrawn, the cells resembled serrated leaves. The cytoplasmic granules were fine and colorless and uniformly distributed throughout the dendrites and cell body. These cells appeared comparable to the incompletely differentiated pigmentless melanocytes found in actively growing black melanomas from spotted-belly *(Sb)* hybrids.

Coarsely granular melanocytes, with evenly distributed granules that had a greenish-red iridescent quality, were also prevalent (FIGURE 30). As compared to the fine-granular melanocytes, the dendrites of the former were fewer in number, wider, and had a blunt appearance. They showed no branching or forking (FIGURE 31). Frequently these cells assumed a round shape with 2 long processes extending from opposite ends. The processes were rapidly withdrawn, so that the majority of coarsely granular melanocytes in a culture eventually were spherical in shape. The fact

FIGURE 29. Finely granular, dendritic amelanotic melanocytes from a living culture. ×570.

that these cells are a later stage in the development of the finely granular, colorless cells was suggested after observing the development of these cells *in vitro*. Frequently cultures were obtained that had only finely granular dendritic cells in the outgrowth zone during the first 2 days. By the third day, the granules became coarser and took on a greenish hue. An increase in size of the granules with increasing age of the cell was also observed in the Cloudman S91 melanoma.[48]

The amelanotic melanocytes were mononucleate or multinucleate; the majority were binucleate cells. The number of nucleoli in each of the nuclei was not uniform.

An organization of melanocytes occurred in the outgrowth zone of a profusely growing culture. The cells clustered together at several points in the medium in such a way as to form nodules (FIGURE 32). It is interesting to speculate on the possibility that new areas of active growth *in vivo* may originate in this manner.

Macrophages. The first cells to reach the outgrowth zone were the macrophages containing a variety of granules and globules. Giant-sized round macrophages 40 to 100μ in diameter were prevalent. When not completely gorged with cellular debris, a clear, advancing pseudopodium was visible, while the engulfed matter occupied the posterior end of the cell. The nucleus was located in the clear zone. The ingested contents consisted of smooth, homogeneous brown bodies, refractile globules with fine brown granules adhered to their surface, and clumps of pigment in shades of brown, yellow, orange-red, and black. The other characteristics of the macrophages in amelanotic melanomas were similar to those in melanomas.

In cultures of a necrotic, slow-growing dorsal fin tumor of the *Sd* albino hybrid, macrophages of different sizes were almost the only cell type present. Slow-growing pigment tumors of mice are similar in this respect. They are much more necrotic and contain many more phagocytes than the rapidly growing tumors.[74] Phagocytosis is a normal process instrumental in the elimination of cells that are degenerating. The presence of macrophages in the necrotic tumor *in vivo* may account for their presence *in vitro*.

Fibrocytes. Thin, spindle-shaped cells with 1 or 2 oval nuclei were present in the outgrowth zone on the first and second days *in vitro*. The cytoplasm was usually clear and colorless but, occasionally, round in-

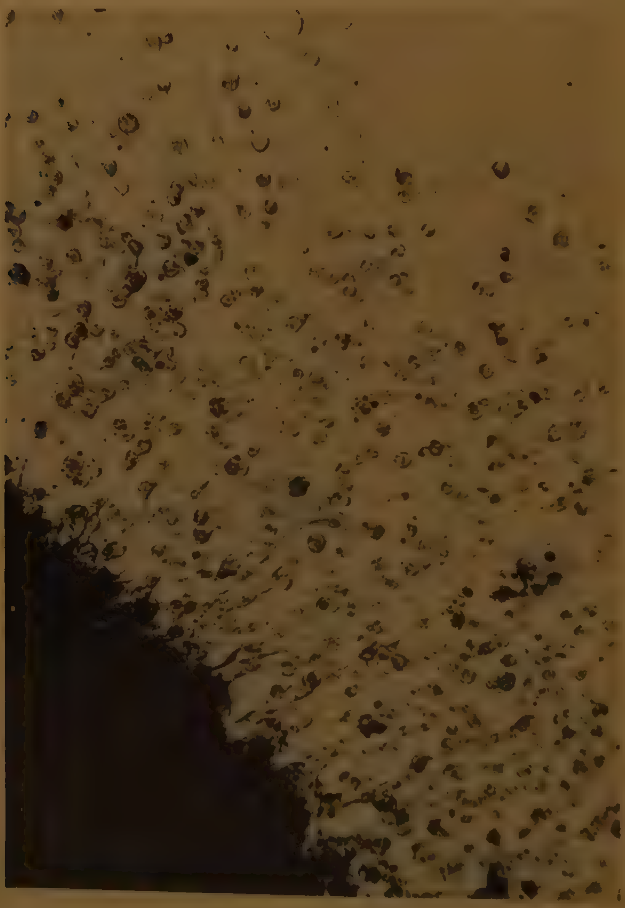
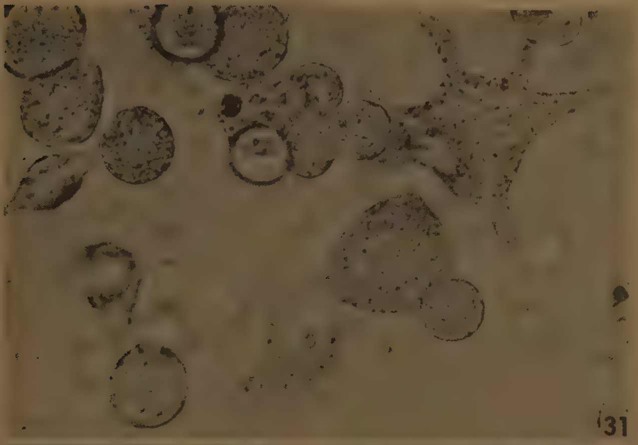


FIGURE 30. Living 2-day culture from an amelanotic melanoma, showing extensive proliferation of the melanocytes with coarse granules. ×120.



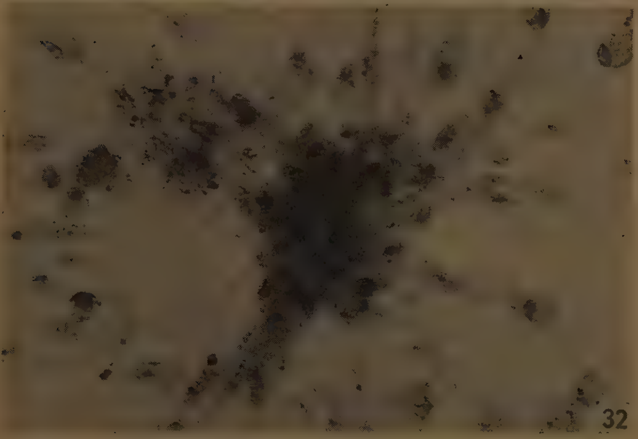


FIGURE 31. Living amelanotic melanocytes with coarse, reddish-green, evenly dispersed granules. ×570.

FIGURE 32. Cluster of amelanotic melanocytes in the outgrowth zone of a culture. ×470.

clusions were present that were similar to the greenish, refractile globules normally present in epithelial cells. On the whole, fibrocytic growth was scanty in most of the cultures.

Epithelial cells. Flat epithelial sheets similar to those in the typical melanoma cultures were prevalent on the first day *in vitro.* Parts of sheets became detached from the explant and formed individual islands of growth some distance away from the explant. These cells were excellent material for cytochemical tests, since there was no interference from the explant or other proliferating cells. The epithelium showed the same characteristic organizations, such as tongue formation, cord or septa

TABLE 2

PERCENTAGE OF CULTURES EXHIBITING 4 CELL TYPES FOUND IN AMELANOTIC MELANOMAS

Tumor and pedigree of hybrid	*Melanocytes*	*Epithelial sheets*	*Melanophores*	*Fibrocytes*
Ni 436, nigra	48%	20%	3%	10%
Ni 437	57	52	8	3
Ni 435				
pigmented region	69	50	33	14
white region	0	33	33	66
Ni (435)2	31	51	7	1
Ni (435)3	63	96	12	0
Ni 440	34	26	16	16
Sdi 382, spotted dorsal	13	0	13	13
Sdi 435	29	0	57	0

formation, cytoplasmic rings or lamellae, and pearl-like growths as observed in black melanoma cultures. In many cultures, the actively growing epithelium appeared to inhibit melanocyte growth. Frequently it was not until the epithelial sheets became detached and moved away from the explant that the melanocytes appeared and, finally, they replaced the epithelial cells as the predominant cell type.

Brown globules were often present in the epithelial cells along with the more usual greenish, refractile globules. These pigmented bodies undoubtedly contribute to the over-all color of the tumor. An occasional globule was observed that was yellow, and sometimes red granules adhered to its surface. Black-coated globules, which occur in black melanoma epithelium, were never seen.

Comparison of growth characteristics in tissue culture. The frequency with which 4 cell types developed from different fragments of each tumor is indicated in TABLE 2. It may be observed that melanocyte growth from dorsal fin tumors of spotted dorsal-albino hybrids *(Sdi)* was never as vigorous as from caudal peduncular tumors of nigra-albino hybrids *(Ni)*. In addition, epithelial growth was not stimulated in the dorsal fin tumors. It is interesting to note the large percentage of explants that contained melanophores in the *Sd* 435 animal. This was a young fish approximately one third the size of the others. Its melanophores were coal-black and stellate.

The white fragment of a gray and white tumor had a preponderance of small fibrocytes, but no melanocyte growth. The tumor cells were arrested in this area, while the stroma cells continued to proliferate. This tissue was similar to the white, nodular, scar tissue present in black melanomas. On the other hand, the pigmented region of the same tumor exhibited vigorous melanocyte growth.

As in the typical melanoma cultures, there was evidence of phagocytosis of erythrocytes by the amelanotic melanocytes and by the epithelial cells.

Cytological Properties

The absence of heavy pigmentation in amelanotic melanoma cultures greatly simplified the task of interpreting the cytochemical and the differential staining reactions, especially those reactions that depend on the formation of black or brown end products. The obscurities caused by melanin in melanoma cultures were twofold. In addition to the problem of distinguishing the reaction products from natural melanins, pale-colored stains and precipitates were often obscured in those cells that were heavily laden with pigment.

The cytoplasm of the amelanotic melanocytes exhibited basophilia on staining with hematoxylin and eosin. The nuclei were not unusual in their staining reactions. Most of the nucleoli had an eosinophilic center surrounded by a dark blue rim. The coarse granules, which had a reddish-green, iridescent quality in the living state, assumed a brown color on fixation. The intensity of the brown color varied in different cells, so that dark brown and light brown melanocytes were always present in the same culture. The extremely fine, colorless granules of the delicate, dendritic cells remained colorless after fixation. Inclusion bodies, which are not ordinary constituents of these cells, were sometimes present in the cytoplasm among the evenly dispersed colorless granules. Some inclusions were round, brown, and granular, similar to the brown bodies normally present in the epithelial cells. Others were erythrocytes that had dark blue nuclei and pink cytoplasm when stained with hematoxylin

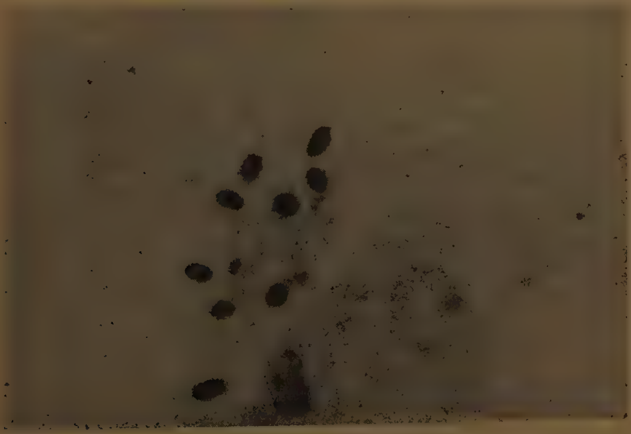


FIGURE 33. Melanocyte with engulfed erythrocytes. Hematoxylin and eosin stain. ×1330.

and eosin (FIGURE 33). Pink-stained inclusions within vacuoles were also present, in addition to solid, blue-stained clumps of pyknotic chromatin. Amelanotic melanocytes consistently showed more evidence of phagocytosis than melanotic melanocytes.

The cytological and cytochemical reactions of the epithelial sheets in amelanotic melanoma cultures were essentially similar to the reactions demonstrated in the epithelial cells of melanomas. Therefore only differences and additional observations are presented. The brown bodies frequently observed in the living epithelial cells of amelanotic melanomas were refractory to dyes. The pigment assumed 2 forms: (1) individual brown granules adhered to the surface of the globules, or (2) the pigment formed a smooth, brown, dense coating that appeared to be part of the spheroid itself. The center of the globule was often empty. Round cells containing brown bodies were numerous in amelanotic melanoma cultures. They appeared to function as macrophages, since they usually contained bits of cellular debris as well as whole erythrocytes. One such typical cell, stained with hematoxylin and eosin, contained 15 small blue erythrocyte nuclei, 2 brown bodies, and 1 pink globule. The conversion of epithelial cells into macrophages is strongly suggested in these cultures. Weiss[75] reported the transformation of spindle cells into macrophages *in vitro,* and concluded that the phenomenon may be peculiar to conditions within tissue cultures. The nuclei of these phagocytic cells were forced into various bizarre shapes and into eccentric positions as the cells became gorged with debris. Frequently the excessive pressure indented the nuclei to the point of fragmentation.

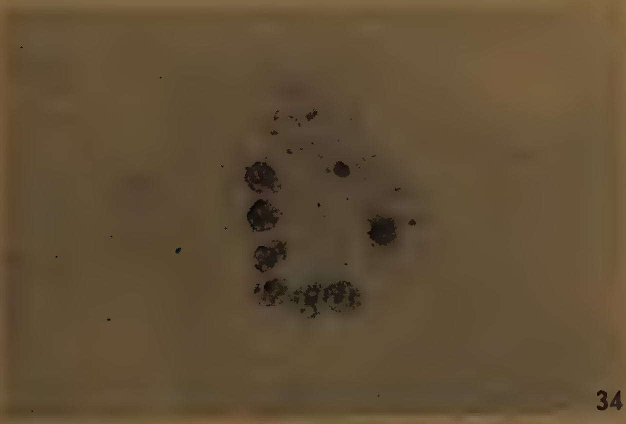
The formation of multinucleated giantlike cells from epithelial cells that did not undergo cytokinesis was also apparent. In the epithelial-like cell shown in FIGURE 34 the similarity of the nuclei to those of the epithelial sheet cells may be noted. Several nuclei are teardrop shaped, as if they had recently pulled apart. Two small brown bodies are present in the cytoplasm. Epithelioid cells that were observed to hypertrophy and form giant cells with 2 to 50 nuclei have been reported in cultures of teleost blood.[76,77]

An epithelial pearl, stained with hematoxylin and eosin, revealed an abundance of large pink-stained irregularly shaped bodies at the core of the pearl. One pink mass was free in the culture, but close to the pearl. Similar eosinophilic masses were present within the cells, and they appeared to be made up of fused refractile globules. The nuclei within the pearl cells were frequently pyknotic (FIGURE 35).

Cytochemical Properties

Nucleic acids. The reactions of the various cell types in amelanotic melanoma cultures to the methyl green-pyronin stain, the Feulgen reaction, and May-Grunwald-Giemsa stain were similar to the reactions of the corresponding cell types in typical melanoma cultures. Extraction of DNA with hot trichloracetic acid was faster in the amelanotic melanoma nuclei than in the nuclei of melanoma cells. The process was completed in one-half to one minute. This may be an indication of the grade of malignancy, since DNA is thought to exist in a low state of polymerization in malignant nuclei.[78] Also, the first major change in the chromatin of degenerating cells is a decrease in polymerized DNA.[12]

Refractile bodies. The reactions of the refractile bodies of the epithelial cells of amelanotic melanomas to the methyl green-pyronin stain, Feulgen technique, polysaccharide tests, lipid reagents, extraction tech-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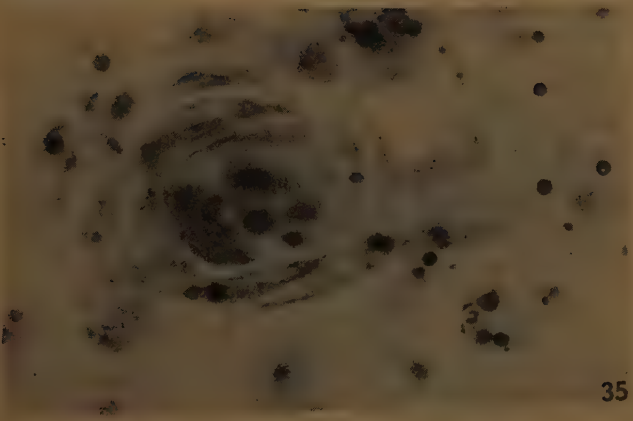
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FIGURE 34. Multinucleated cell from an amelanotic melanoma culture. Hematoxylin and eosin stain. ×1330.

FIGURE 35. Pearl-like formation in an epithelial sheet from an amelanotic melanoma culture. Note pyknotic nuclei, methyl green-pyronin stain. ×1330.

niques, and the performic acid-Schiff method were similar to the results obtained with the epithelial cells of melanoma cultures.

Glycogen and lipids. The reactions of the amelanotic melanoma cells to the periodic acid-Schiff test (PAS) for glycogen and the lipid tests were similar to the reactions of the typical melanoma cells.

Thiol groups. The amelanotic melanocytes reacted positively to both the ferricyanide method of Chèvremont and Fredéric and to the azo dye method of Barrnett and Seligman for thiol groups. With the ferricyanide method, the nuclear rim and nucleoli of most cells stained a darker blue than the cytoplasm, chromatin, and nucleoplasm, but there were also melanocytes in which the cytoplasm reacted more strongly than the nucleus. The individual colorless granules of amelanotic melanocytes were blue.

Epithelial cell nuclei were also blue, but they did not stain as deeply as melanocyte nuclei. As in the melanoma cultures, the refractile globules were not consistent in their reaction. Controls pretreated with iodoacetic acid gave results similar to those reported for melanoma cultures.

With the more specific azo dye method, the cytoplasm and nuclei of the melanocytes appeared to stain with the same intensity, although occasionally the nucleolus appeared a slightly darker blue. The blue color of the cytoplasm was localized in the granules, being most intense near the nucleus. Its intensity decreased toward the periphery of the cell (FIGURE 36).

In the epithelial sheet cells, the nucleoli and nuclear membranes were blue with the azo dye method. The nucleoplasm stained reddish-

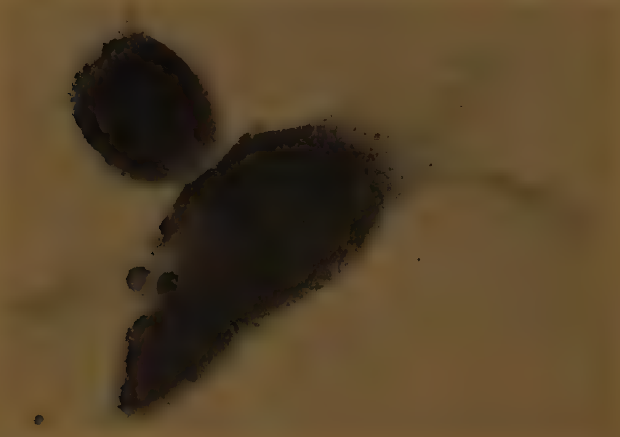

FIGURE 36. Amelanotic melanocytes treated with Barrnett and Seligman's thiol technique. Note the cytoplasmic as well as the nuclear activity. ×1330.

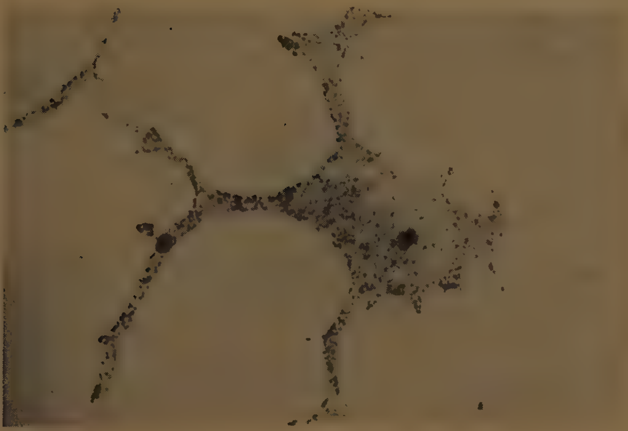

FIGURE 37. Positive argentaffin reaction in an amelanotic melanocyte. ×1330.

blue. The cytoplasm around the nuclei was reddish-blue and became less intense toward the periphery. The refractile globules stained reddish-blue or clear blue. The circular lamellae were blue. The blue color is indicative of a greater concentration of thiol groups than the reddish hue. The reaction was successfully inhibited with iodoacetic acid in control cultures.

Alkaline phosphatase. Phosphatase activity was demonstrated in the nuclei and cytoplasm of the amelanotic melanocytes. Black granules were scattered in the cytoplasm, which gave the cells an over-all gray appearance. The nuclei were outlined with a heavy black precipitate, and the nucleoli and chromatin were black. In occasional melanocytes only the cytoplasm reacted, but not the nuclei. Comparable cytoplasmic activity was difficult to demonstrate in the black melanoma cells, because of the interference of the pigment granules. Phosphatase activity was present in the nucleus and cytoplasm of the epithelial sheet cells.

Pigment reactions. Although there are no specific chemical tests for melanin, the brown granules in the cells of amelanotic melanomas reacted in a similar way to melanin. They were bleached rapidly by concentrated nitric acid during the first few minutes of exposure. Chromic acid, likewise, removed the brown color and toned the explant tissue to a golden yellow. Potassium permanganate removed all color so that the granules and tissue were pure white. A 10 per cent peroxide solution was extremely effective in decolorizing the brown pigment. There was no loss of color with N HNO_3 or with chlorine.

The peroxide bleach is considered the most significant for melanin, since other pigments, such as lipofuscins, resist decoloration for long periods in this oxidant.[17] The other oxidants bleach many pigments at the same rate as melanin.

The brown pigment also resembled melanin in its reaction to silver. This was made evident using Masson-Fontana's technique,[17] in which ammoniacal silver nitrate was reduced to metallic silver. The tan-colored explants became coal-black, the melanocyte pigment granules dark brown or black, and the clumps of brown melanin and the brown globular bodies in the macrophages were strikingly black. Similar results were obtained with the argentaffin reaction of Tandler.[30] The very fine, completely colorless granules of melanocytes were colored a dark, reddish-brown or black, and they became larger and coarser (FIGURE 37). Differences in color may be indicative of differences in reducing capacity of individual granules.

In addition to being argentaffin, melanin granules are characteristically argyrophil, as they can be impregnated with silver. With Cajal's silver impregnation technique, the melanocyte granules became golden brown to black. The darkest granules were usually situated around the nucleus (FIGURE 38). The tan explants were coal-black.

Tyrosinase activity was demonstrated in amelanotic melanoma cell cultures by incubating them in a buffered dopa substrate. The whole explant, the pigmentless melanocytes, and the epithelial sheet cells became brownish-black. In some cells the nuclei and surrounding cytoplasm showed more activity than the peripheral cytoplasm. The round, nondendritic melanocytes were a darker brown than those with extended processes (FIGURE 39). The positive reaction in the epithelial cells was not as intense as in the melanocytes. Frequently the 2 nuclei of a binucleate epithelial cell did not react similarly, as one stained dark brown and the other pale tan. The sculptured lamellae and a few of the refractile bodies stained brown.

When tyrosine plus a dopa catalyst were used as a substrate, enzyme activity was evident. Tyrosine, alone, did not stimulate tyrosinase.

The results of inhibition experiments proved largely similar to the results obtained with black melanoma cultures. There was complete inhibition with 1N HCl. Pretreatment with phenylthiourea or sodium bisulfite gave no inhibition, but when used as competitive inhibitors in the substrate medium, inhibition was complete. Comparable inhibition results were obtained when the substrate was dopa or tyrosine plus the dopa catalyst.

Negative pigment reactions. In further determinations of the nature of the pigment present in the amelanotic melanomas of homozygous albino fish, negative reactions were obtained for the following substances, using the recognized techniques for their demonstration: (1) hemosiderin —

ferrocyanide method of Perls; (2) hemofuscin — basic fuchsin and hematoxylin method of Mallory; (3) ceroid — OsO_4 bleaching methods and acid-fast technique; (4) ascorbic acid — acid silver nitrate technique; and (5) incubation in buffered potato tyrosinase.

COMPARISON OF TYPICAL MELANOMAS AND AMELANOTIC MELANOMAS

Living Cultures

Several significant similarities and differences were found in the cells of melanomas from pigmented and albino hybrids growing *in vitro:*

(1) The number of cell types in the cultures of amelanotic melanomas

FIGURE 38. Positive argyrophil reaction in an amelanotic melanocyte. ×1330.

FIGURE 39. Dopa activity in amelanotic melanocytes. Note the decreasing activity toward the periphery of the cells. ×570.

appeared to be greater at first than in melanoma cultures. This difference, however, may be attributed to the atypical shapes assumed by the amelanotic melanocytes, which varied from amorphous cells to highly dendritic, typical melanocytes.

(2) The granules in amelanotic melanocytes were colorless, brown, or had a reddish-green iridescence, and were uniformly distributed throughout the cells. The granules in melanotic melanocytes were brown or black, and were frequently concentrated in the dendrites or at the serrated periphery of the cells.

(3) The black melanophores occasionally present in amelanotic melanoma tissue were usually punctate or lysed. They were more frequent, well-formed, and stellate in melanoma tissue.

(4) Fibrous nodules were present in amelanotic melanomas as well as in black melanomas.

(5) Melanomas in terminal stages resembled amelanotic melanomas in tissue culture. In each, the proliferating cells were pigmentless, not well-differentiated melanocytes. They were frequently amorphous, or they had difficulty in maintaining the typical dendrites.

(6) The globules in melanoma epithelium often had a black coating. Those in amelanomas were either without pigment or occasionally were brown.

(7) The pigment cells of amelanotic tumors rarely exhibited the degree of differentiation reached by melanotic melanocytes.

(8) Generally, amelanotic melanomas grew more profusely in tissue culture than melanomas.

Fixed Cultures

In considering the results of the cytochemical and staining reactions on fixed cultures, several features are evident.

(1) The pigment present in the amelanoma cells is a form of melanin.

(2) The pigmented and nonpigmented melanocytes have melanogenic enzyme activity.

(3) Thiol groups are present in the amelanotic pigment cells, but they are absent from the melanotic pigment cells.

(4) The localization of thiol groups *in situ* coincides with the localization of the melanogenic enzyme *in situ*.

(5) The characteristics of the pathologic process are generally more prominent in amelanotic pigment cells than in pigment cells of melanomas. There are more foreign-body inclusions in the former.

(6) The epithelial cells of both the melanomas and amelanotic melanomas contain pigment granules. Both have melanogenic enzyme activity.

The results of the relevant cytochemical tests, staining methods, and

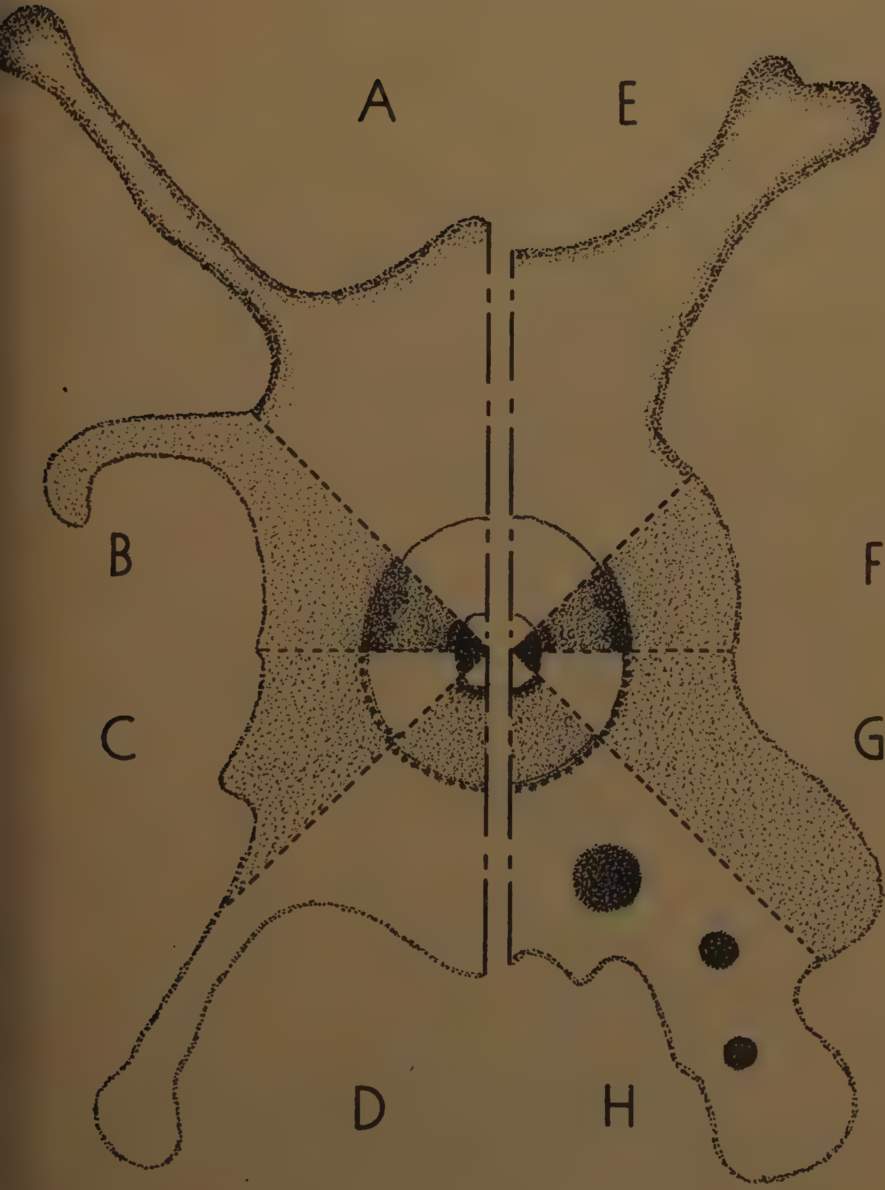

FIGURE 40. Comparison of some of the cytochemical localizations in the melanotic melanocyte *(ABCD)* and the amelanotic melanocyte *(EFGH)*. The relative intensities of the reactions are indicated by the density of the stippling. *A* and *E* show the localization of glycogen as determined by the periodic acid-Schiff reaction; *B* and *F*, the distribution of alkaline phosphatase activity; *C* and *F*, the distribution of pyroninophilic substances; and *D* and *H*, the distribution of substances stained by methyl green or by the Feulgen reaction.

pigmentary reactions on the pigment cells of melanomas and amelanotic melanomas are summarized in FIGURES 40 and 41.

Discussion

LIVING CULTURES

The neoplastic nature of melanomas in xiphophorin hybrids is indicated in part by their prolific growth in tissue culture and by the extensive liquefaction of the solid plasma medium. Normal tissue from similar animals does not grow readily,[1] nor does the newly formed overgrowth. It appears that the tumor grows slowly *in vivo* until a critical point, after which its growth *in vitro* is extensive. This is in accord with Berenblum's[79] theory of carcinogenesis in which he postulates a "chain reaction" mechanism that is not in effect until a critical number of dormant tumor cells accumulate. Thus a cell colony of a critical size is necessary for independent, progressively increasing growth.

There is a difference in the proliferative character of melanomas obtained from the various sites. When situated in a region that normally has a good vascular supply, the tumor grows well both *in vivo* and *in vitro*. Where the blood supply is scant, as in the dorsal fin, parts of the tumors are generally necrotic and are sloughed off. The relatively poor growth of dorsal fin tumors in culture may be related to the previous degenerative processes going on *in vivo*. There is a stage in development of the dorsal fin tumor, however, when the blood supply appears to be adequate for the proliferating cells and cultures yield good growth.

In a comparison of histological sections of dorsal fin melanomas with melanomas on the body proper, Levine[3] concluded that the former are benign, superficial, fibrous overgrowths, made black by the invasion of macromelanophores and macrophages, while the latter are malignant tumors composed of fixed tissue cells, melanocytes, with endogenous melanin. In the present investigation, however, fibrous tumors were found in areas of the body other than the dorsal fin. These tumors yielded only a sparse growth of small spindle cells in culture. On gross inspection, the fibrous mass was usually white, but microscopic examination revealed scattered, disintegrating melanophores, and varying numbers of melanocytes, depending upon the stage of development of the tumor. Regardless of the site of the tumor, the connective tissue stroma seems to persist for a longer time than the pigment cells when the vascular supply is no longer in balance with the increased growth. Finally, the fibrous area is also sloughed off.

A fish with a fibrosed tumor frequently may develop a second actively growing tumor adjacent to, or not far separated from, the fibrous mass.

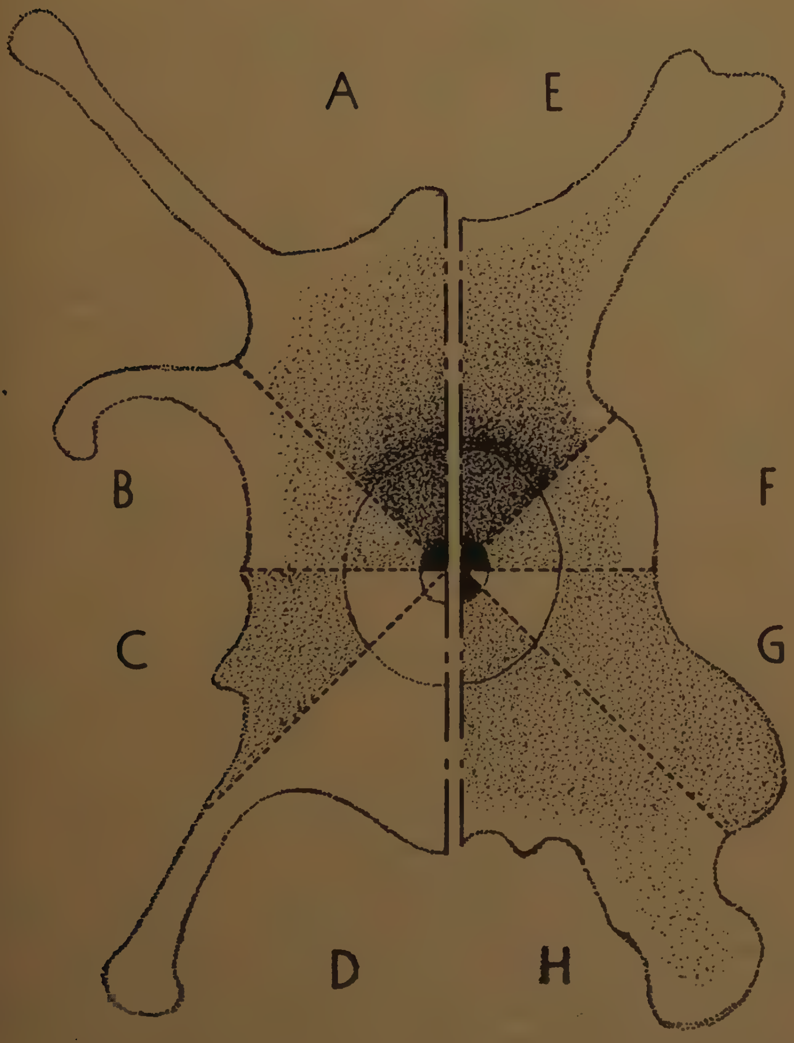

FIGURE 41. Comparison of melanogenic enzyme activity and certain pigment reactions in melanotic (*ABCD*) and amelanotic (*EFGH*) melanocytes. The relative intensities are indicated by the density of the stippling. *A* and *E* show the distribution of dopa oxidase activity (*dl*-dopa), and *B* and *F* show the tyrosinase activity (*l*-tyrosine and *dl*-dopa catalyst). There is no activity in the presence of *l*-tyrosine alone. Melanogenic enzyme activities are subject to general and specific inhibitors. Both are inhibited competitively by phenylthiourea. *C* and *G* show various melanin reactions: argentaffin, argyrophil, bleaching by oxidants, and accumulation of radio-*l*-tyrosine (2-C^{14}). Negative pigment reactions in amelanotic melanocytes include those for hemosiderin, hemofuscin, and ceroid. *D* and *H* show the distribution of thiol groups. Note the intense participation of nucleolus in the tyrosinase and dopa oxidase activities. The distribution of thiol groups in amelanotic melanocytes parallels the distribution of melanogenic enzyme activity.

The genetic determination for atypical pigment-cell growth persists in spite of the failure of the first potential tumor. A genetic composition that favors tumor formation does not appear to be sufficient, in itself, for the successful growth of a tumor. To reach its full potential, the proper environmental and other conditions must also be present.

Cultures of rapidly growing fragments have a greater number of poorly differentiated melanocytes than the slow-growing cultures. The cells may be characterized by a sparsity or complete lack of pigmented granules, small size of the granules, and inability to maintain the typical dendritic shape. Active melanocyte growth *in vivo* can be recognized by a decrease in pigmentation in the tumor, apparently caused by an increase in the number of immature cells. Such pigmentless areas are quite different from the inactive, colorless, fibrous masses indicative of a cessation of pigment-cell growth. The active tumors are soft, edematous, and translucent in contrast to the hard, white, opaque, scarlike appearance of the inactive ones. In human melanoma, the absence of pigment is observed in some of the most rapidly growing and malignant cases; heavily pigmented primary tumors may give rise to pigment-free metastases.[80]

The observations of various investigators[40, 50, 81] that the different types of pigment cells, melanoblasts, melanocytes, and melanophores of xiphophorin hybrids are actually progressive stages in the differentiation of a single cell type, appear to be confirmed by these experiments. Likewise, in *Thalassoma bifasciatum*, the bluehead maturation of pigment cells was noted in regeneration experiments. Faintly pigmented cells with delicate processes changed to stellate cells in which the color of the pigment granules gradually increased in intensity until the mature state was attained.[82] The well-differentiated melanocytes of slow-growing xiphophorin tumors were associated with great numbers of melanophores, and the tissue was coal-black. On the other hand, active tumors with immature melanocytes had few melanophores. Furthermore, melanophores exhibit the characteristics of well-differentiated cells in tissue culture, insofar as they do not reproduce and ultimately become senescent and die.

Other tissues closely associated with the pigment-cell tumors include a hyperplastic epithelium and supporting fibroblasts. Hypertrophy of associated tissues is thought to be due to a growth-promoting substance released by the tumor cells, or due to the products of autolysis of necrotic cells.[83, 84] The epithelium of fish melanomas is especially interesting, since it infiltrates the tumor and grows readily in culture. Cowdry[85] is of the opinion that as long as epithelial cells remain normal, their invasiveness is nil, and that the carcinomatous cells to which they give rise are invasive. Epidermal extensions into tumor tissue have also been reported in a case of xantho-erythrophoroma in the platyfish.[81] In human pigment-cell tumors, the histological distinction between benign nevus of childhood and juvenile melanoma is the epidermis, which is thin in the benign tumor and hyperplastic and ulcerated in the melanoma.[86]

In the present experiments, active epithelial-cell proliferation *in vitro* usually accompanied actively growing melanomas. The cells formed pearls, cords, and tongues. The pearls resembled the "keratin pearls" formed in squamous-cell carcinomas. Normal, nonglandular, human epithelium grown in tissue culture does not form pearls.[87] From the observations made in this investigation, it appears necessary to consider the epithelial tissue in the histogenesis of pigment-cell tumors of fish.

The delicately sculptured concentric markings that form an intricate pattern in some of the epithelial sheet cells were first noted by Dederer[88] in epithelium derived from *Fundulus* fish embryos grown *in vitro*. Goodrich and Biesinger[82] also reported this phenomenon in sections of skin from another fish, *Halichoeres*. Using phase-contrast optics, Zollinger[89] observed a delicate pattern of wrinkles, resembling a fingerprint, on the surface of squamous epithelial cells from the human cheek. He attributed the phenomenon to folds in the superficial layer of the cellular membrane, interdigitating with those of adjacent cells. The markings also resemble the submicroscopic lamellae or endoplasmic reticulum seen with electron micrographs in cells from various tissues.

Since the lamellae in the fish epithelium were never present along with the refractile globules, a relationship between secreted globules and the lamellae might possibly exist. If there is a joining of the surface boundaries of the extracted globules, a pattern of concentric rings could conceivably be formed. Surface membranes on intracellular bodies, responsible for osmotic effects, were demonstrated in cephalopod neurons by Young.[90] The membranes also serve to form isolated compartments within the cytoplasm. Zollinger[91] demonstrated a thin, black, wrinkled membrane or shell with the phase microscope that remained after cytoplasmic granules were extracted. It has been suggested that intracellular-cytoplasmic membranes may represent a fundamental principle of organization of the cytoplasm.[92,93] This could be responsible for lamellae formation on a microscopic as well as a submicroscopic basis.

CYTOCHEMICAL ANALYSIS OF FIXED CULTURES

The results of cytochemical tests indicate that some of the constituents of the cells in the melanoma are of general distribution, as in most tissues, because they are concerned with metabolism. Examples of such substances are alkaline phosphatase, DNA, and RNA. A postulated function of phosphatase is that it is needed in cell division and protein metabolism, as it facilitates both the splitting and synthesizing of RNA and DNA.[94,95] Since the tumor cells proliferate rapidly in tissue culture, high phosphatase activity should be expected. In fact, when the phosphatase activity of human lymph nodes cultivated *in vitro* was compared with tissue sections of the same tissue, more activity was demonstrated in the proliferating cells.[96] The localization of enzyme activity, mainly in the nucleus and nucleolus in fish melanomas, supports the suggested function of phosphatase, since here it is in association with DNA and RNA. This distribution corresponds with the findings of Hull[97] and Chèvremont and Firket,[98] who established a correlation between enzyme activity and the mitotic index in tissue cultures of tumor cells. The strength of the nuclear reaction parallels the amount of outgrowth. Furthermore, in experimental carcinogenesis, the increase in alkaline phosphatase activity of the nucleus is one of the first changes to be observed with proliferation. Increased phosphatase activity in the nucleus was also demonstrated in nontumorous, regenerating rat liver.[99]

Other chemical constituents that were identified are associated with specific cell types in the tumor tissue, and they are normally concerned with the specialized processes carried on by these cells. In the epithelial cells of fish melanomas, thiol groups and keratin were localized. The solid or semisolid nature of the refractile inclusions, or at least of their limiting membranes, is suggested by the irregular and doughnut-shaped forms that these bodies often assume. Moreover, an attempt to burst globules by puncturing them with a microneedle was unsuccessful. The globules were merely displaced by the pressure of the needle without suffering changes, an indication of rigidity.

Cytochemical tests on the globules revealed no mucins. Since there were only an occasional globule or isolated areas within a globule that reacted positively for lipids, a changing composition that did not permeate the entire body at once was indicated. The reduction of osmium tetroxide by many of the globules proved significant. The unstable compound, OsO_4, reacts with a limited number of substances, approximately in the following order:[100] (1) thiol groups have a direct reducing action; (2) double bonds of unsaturated fats show about the same reactivity; (3) amino groups, hydroxyl, and aldehyde groups in terminal positions are reactive. Carbohydrates and nucleic acids are inert towards OsO_4. Eleidin, a reducing agent formed from keratohyaline, reacts with osmic acid.[56] This is probably due to the thiol content of cysteine, a component of keratohyaline.

Since thiol groups were demonstrated in many of the globules by 2 different methods, the presence of the protein substances, keratohyaline and keratin, was considered. The organization of the epithelium into pearl formations in culture was also suggestive of prekeratin and keratin. The pearls were histologically similar to the keratin pearls frequently present in squamous-cell carcinoma, and they gave the same staining reaction with eosin. Furthermore, keratohyaline granules have a high refractive index,[101] and concentric lamellae formations have been reported in keratinizing epithelium *in vitro.*[102]

The variable reaction of the globules to both acid and basic dyes was also suggestive of a protein that behaves as an amphoteric electrolyte. Staining with acid dyes would thus be effected below the isoelectric point, whereas a combination with basic dyes would occur above this point. There is a shift of isoelectric point with different fixatives.

The lack of uniformity exhibited by the globules in response to chemical tests may be accounted for by the change from prekeratin to keratin. Within the same cell, all the globules apparently do not mature at the same rate. Litvac[102] stresses the inconsistency of the response of keratinizing epithelium to histochemical tests. The principal chemical change that occurs is the oxidation of thiol groups to form disulfide linkages. This may account for the inability of some of the globules to reduce osmic acid and to give a positive thiol reaction. The widespread reaction to the performic acid and Schiff reagents (PFAS) is due to the performic acid that oxidizes both disulfide linkages and thiol sulfur linkages to sulfonic and sulfinic acid groups which, in turn, restore the color to leukofuchsin.[17, 103, 104] Thus both the mature globules that contain disulfide groups and the globules that contain thiol groups would react positively to the PFAS reaction.

The only other substances known to react positively to the PFAS technique are the unsaturated lipids and ceroid pigments.[59] Since lipids and lipid pigments were ruled out by staining and extraction techniques, the positive reaction was ascribed to keratin.

The miscellaneous reactions exhibited by the globules can also be explained on the basis of keratinization. The positive fat reaction given by 1 or 2 globules in a culture or, occasionally, by minute areas within a single globule, may be attributed to paraeleidin, a fatty substance formed from keratohyaline.[101] The reduction of ammoniacal silver nitrate to silver is due to the reducing action of thiol groups. The positive cobalt sulphide precipitate, formed on the globules in both experimental and control cultures during Gomori's alkaline phosphatase reaction, may be attributed to the sulphur released from keratin during incubation in the alkaline solution. The sulphur reacts with cobalt nitrate to form the insoluble cobalt sulphide. The variable but brilliant Feulgen nucleal reaction exhibited by some of the globules may be due to reactive groups revealed by the breakdown of the protein during acid hydrolysis. The peripheral staining, as well as the total staining of the globules with Schiff's reagent is probably a matter of penetrability.

The deposition of glycogen in both the melanocytes and epithelial cells of the fish melanomas may be correlated with an anaerobic form of metabolism. Glycolytic activity has been demonstrated in Cloudman S91 mouse melanoma and in the S91A amelanotic melanoma.[105, 106] There is also an elaboration of glycogen during lymphocyte maturation in lymphatic leukemia and lymphosarcoma,[96] and glycogen has been reported in Yoshida rat ascites sarcoma cells.[97] The significance of the epidermal glycogen in the fish melanomas is uncertain. Glycogen is known to occur normally in human epidermis.[107]

MELANIN

The physical and chemical properties of the granular inclusions in the melanocytes of melanomas are typical of the melanins. The colorless granules of the incompletely differentiated melanocytes also possess many similar characteristics. They reduce ammoniacal solutions of silver nitrate to metallic silver. The extent of the reduction is variable, as evidenced by the brown or black precipitates deposited on the granules. The variability may reflect the developmental stage of the granule. Premelanin granules do not reduce silver salts. Premelanin may be considered to be the granular matrix that has an affinity for the products of melanin synthesis. The matrix is probably a protein, since melanin usually occurs as a melanin-protein complex. Moreover, Kopac[108] has suggested that the protein matrix itself may be involved in melanin synthesis. Unbound tyrosyl groups present in proteins can undergo oxidation in the presence of tyrosinase and produce pigment. Such proteins could form a surface on which further polymerization of melanin would occur. The properties of the colorless or lightly pigmented granules of young pigment cells would therefore vary with the number of labile tyrosyl groups that are available at a particular moment, with the degree of polymerization of the oxidized polyphenols with which a granule is associated, and with the quantity of polymerized end products present on the particular granule.

Some of the colorless granules are argyrophil, a property that Masson[68] believes to be indicative of premelanin. Premelanin granules first appear in the cell body of dendritic cells. Here they undergo maturation and migrate to the extremities of the processes. Smith[109] traced pigment-granule formation in the pigmented epithelium of the embryo chick eye *in vitro* from small, colorless granules in the cytoplasm that increased in size, number, and depth of color until they became black and rod-shaped. He concluded that the chromogen is laid down faster than the coloration takes place. Algard[43] studied the origin of pigment granules in amphibian cells in tissue culture. He believes that small spherules with the same optical appearance as the larger mitochondria might be the emerging pigment granules. Electron micrographs of pigmented melanocyte granules made by Laxer *et al.*[110] reveal them to be composite structures made up of a colorless "premelanin" core upon which the polymerized derivative of oxidized polyphenols is deposited as a sheath or envelope. The exact nature of the matrix could not be determined. Plummer and Kopac[111] observed from *in vitro* studies that a matrix is required for the polymerization of melanin precursors in order that melanin granules may be formed. Such evidence would tend to eliminate the possibility that pigment granules are formed *de novo* from the oxidized polyphenol monomer.

The differences in degree of impregnation with silver of the colorless granules of fish melanocytes during the argyrophil reaction is interesting in view of the distribution. Highly impregnated granules were closer to the nucleus than the lighter ones. This may have a bearing on the controversial question as to the site of melanin synthesis. A nuclear origin has been suggested by some,[80, 83, 112, 113] while a mitochondrial origin has been ascribed by others.[106, 114-116] The latter was refuted when phase contrast and electron microscopy were done on the same tissue.[117] The ultrastructure of the cytoplasm has also been suggested as a site of pigment formation.[83, 108, 118] The conflicting reports may reflect a variable mechanism of melanin synthesis in cells from different sources.

In the fish melanocytes, tyrosinase activity was highest at the periphery of the nucleus and in the nucleolus. Cytoplasmic activity was greatest in that part of the cytoplasm adjacent to the nucleus. The importance of the nucleolus in enzyme production has been cited, and it is also believed to contain proenzymes.[119] Becker *et al.*[120] observed dopa oxidase activity in the form of nuclear caps and at the tips of dendrites in human melanocytes.

The origin of pigment cannot be solved with certainty by cytochemical methods unless diffusion artifacts can be controlled. A step in this direction has been made with the development of a spot-spray technique in which an enzyme-substrate mixture is made to react on filter paper.[121] Future application of this technique to cytochemistry should include dissecting out intracellular components and subjecting them to chemical reactions on filter paper. A more exact localization will then be possible.

The presence of melanin in epithelial cells brings up another controversial question. It is thought by some that melanocytes are the only cells capable of synthesizing pigment. The presence of melanin in epithelial cells is visualized as the result of an active transfer of pigment from the dendrites of melanocytes to the epithelial cells.[122] In the epithelial cells, the melanin becomes arranged in the form of perinuclear "caps." The phagocytic action of epithelial cells toward melanin has also been reported.[123, 124] On the other hand, Smith[109] observed the pigmented epithelium of the embryo-chick eye in tissue culture, and followed granule formation in the cytoplasm of the proliferating cells. The pigment granules became concentrated around the centriole. From a study of benign and malignant moles, the same author concluded that there are 2 types of cells capable of producing melanin pigment. In tissue culture, one grows like epithelium and the other like connective tissue.[118] The epithelial cells that develop in tissue cultures of adult human epidermis, both Negro and white, contain varying amounts of pigment granules.[125] In addition, the thesis has been advanced that epithelial cells are not only capable of melanin formation, but they may be transformed into melanocytes and melanocarcinomas.[113, 126] From observations of embryonic teleostean grafts, Oppenheimer[127] concluded that under certain experimental conditions pigment cells may differentiate from cells that normally do not contribute to the teleostean counterpart of the neural crest. Cells other than the usual ones can take over the function of pigment-cell formation.

The melanin within epithelial cells of fish melanoma is localized on the globules, which appear to serve as a matrix for the pigment. This orientation is constant, and it does not suggest phagocytized melanin. It is generally recognized that ingested material takes the form of masses of varying sizes and shapes. In fact the presence of dispersed or clumped granules is a method of distinguishing between pigment-producing and pigment-carrying cells. Furthermore, phagocytized inclusions are usually surrounded by vacuoles. The issue of epidermal pigment is not completely solved, and it awaits improved methods.

The positive reactions of epithelial cells to tyrosine and dopa may be interpreted in several ways: (1) tyrosinase is present in epithelial cells; (2) the amino acid is taken up for protein synthesis and becomes colored by autoxidation; or (3) the positive reaction is due to the presence of cytochrome oxidase, which is known to oxidize polyphenols. Preliminary studies involving incubation with radioactive tyrosine* and subsequent radioautographs indicate a definite and rapid uptake of the amino acid by the epithelial sheet cells. According to Bradfield,[94] epidermis, hair matrix, some mucous membranes, and some blood cells remain dopa-positive in the adult. If tyrosinase is considered to be present in epithelial cells, another factor, an antioxidase or inhibitor of tyrosinase, must be postulated to account for the variable presence of melanin in some melanoma epithelium but not in all.

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*Lot No. 3-244-9, Tracerlab, Inc., New York, N. Y., specific activity 0.56 mc./mg., Atomic Energy Commission Authorization No. Ca-28773 to M. J. Kopac.

The tyrosinase in human skin normally exists in an inactive or inhibited form. It must be irradiated with ultraviolet light, X rays, or thorium X to destroy the inhibitor before enzyme activity can be demonstrated.[120, 128, 129] Figge[130] presented further evidence for an inhibitor of tyrosinase activity by *in vitro* experiments using cysteine and reduced glutathione, which contain thiol groups, to prevent the enzymatic oxidation of tyrosine. Thiol groups are believed to bind the copper enzyme, tyrosinase, and thus prevent melanin formation.

Thiol groups were present in the fish melanoma epithelium. Quantitative differences of thiol possibly may account for the presence or absence of pigment in the epithelium of the various hybrids used. Even in pigment cells, enzyme activity is frequently variable, and this may be a function of the amount of inhibitor present. Kertész[131] measured the activity of 4 human melanomas that were histologically identical, and he found variations from completely negative to very high dopa oxidase activity. Figge[132, 133] was able to stimulate melanogenesis in normally nonmelanin producing cells of tadpoles by shifting the redox potential to a more favorable level. In the presence of indophenol dyes there was a synthesis of melanin in the connective tissue cells. It is possible that the contradictory reports in the literature concerning melanin synthesis, in cells other than true pigment cells, may be due to different conditions under which the cells were observed, with reference to the redox potential.

ANALYSIS OF PIGMENT CELLS FROM MELANOMAS OF ALBINO HYBRIDS

The presence of pigment in the melanomas of homozygous albino hybrids is a problem that has received much speculation. The autonomous nature of melanocytes and chromosomal changes in these cells have been suggested to account for the production of visible pigment.[41] The possibility that the pigment is a substance other than melanin, and that it might be associated with the metabolism of the tumor, was considered in the present investigation. The results of chemical tests for morphologically similar chromogens, however, such as hemosiderin, hemofuscin, and ceroid were negative. Instead, the pigmented granules exhibited the properties of melanin.

Since the majority of pigmented granules are in shades of brown or tan, they differ from the black pigment granules in nonalbino animals. Although the albino gene is unable to suppress pigment production in the tumor cells, its influence is definitely evident in the lighter color of the individual pigment granules. Macroscopically, the tumors of albinos frequently appear grayish-black but, actually, brown pigment cells grouped together in dense masses produce the darker effect.

Genes for albinism may act through one of several processes: (1) by causing an insufficiency of chromogen; (2) insufficient enzyme; or (3) by means of an antioxidase or inhibiting process. Goodrich[134] suggested that a lack of chromogen might be the difference between colorless and colored melanophores of *Oryzias*. Amelanotic fragments of Harding-Passey and Cloudman S91A mouse melanomas were found to be deficient in enzyme activity.[135,136] In the present experiments, melanogenic enzyme activity was demonstrated in the amelanotic melanocytes by cytochemical tests. In addition, preliminary studies with radioactive tyrosine indicate a definite uptake of the labeled amino acid by the amelanotic melanocytes. Since autographs were obtained in one day, the amount of radioactive tyrosine incorporated appears to be high. In spite of this, the color of the cultures was not different from controls that had not received the chromogen. In view of these findings, it may be postulated that the albino gene of *Xiphophorus helleri* functions as an inhibitor preventing the formation of oxidized, completely colored melanin.

The inhibitory mechanism may operate through the reducing action of thiol groups that have been demonstrated in amelanotic melanocytes in the present investigations. Comparable groups were not present in the melanocytes of melanomas in nonalbino fishes. It is interesting to note that the *in situ* localization of thiol groups parallels the distribution of tyrosinase. This suggests that the amount of pigment present in the amelanotic cells may depend upon a state of equilibrium between the 2 antagonists. Melanin is a reversible oxidation-reduction system that is tan in the reduced state and changes on oxidation to brown, dark brown, and black.[133,137] Pallor has been produced experimentally by shifting the redox potential in the melanophores of amphibian larvae with indophenol dyes.[132] A shift in either direction inhibits the enzyme. In mottled rabbit skin, a quantitative balance has been demonstrated between tyrosinase and thiol compounds, the degree of inhibition of melanin formation varying directly as the log of the molar concentration of thiol groups.[138]

In the normal pigment cells of albino fish, melanin pigmentation is successfully suppressed by the albino gene. In the melanomatous cells of albino hybrids, however, the inhibitory action of the albino gene may not be capable of keeping pace with the rapid proliferation of the stimulated melanocytes. Pigment may be formed faster than it is reduced. The delayed action of the albino gene was noted by Breider,[49] who obtained newborn, pink-eyed, homozygous albino, xiphophorin hybrids that were colored black. Gradually the black color faded, so that some of the animals resembled ordinary albinos. This mode of action infers that thiol groups may function after mature pigment granules are formed and that, subsequently, the granules are reduced to the leuko state.

Other modes of action must also be considered that might possibly lead to the formation and maintenance of colorless granules in melanocytes of albinos. The series of reactions by which pigmented granules are synthesized, as established by Raper, and as illustrated below, are generally accepted.[139-143]

$\text{Tyrosine} \xrightarrow{\text{tyrosinase}} \text{Dopa} \xrightarrow{\text{tyrosinase}} \text{Dopa Quinone} \xrightarrow{\qquad}$

$\text{Leukodopachrome} \xrightarrow{\qquad} \text{Dopachrome} \xrightarrow{\text{autoreduction}} \text{5,6 Di-}$

$\text{hydroxyindole} \xrightarrow{\text{oxygen}} \text{Indole 5,6 Quinone} \xrightarrow{\text{polymerization}} \text{Melanin}$

The nature of the agglomeration of indole nuclei to form colored melanin granules is not well established.

Deviations in the scheme of reactions, as a function of the albino gene, might occur in one of several stages and bring about colorless granules. Different inhibitors are known to function at different steps in the reaction. Thus hydroquinone blocks the oxidation of tyrosine to dopa, but it does not have an inhibitory action on dopa. It can only function at the first stage in melanogenesis.[144] Ascorbic acid, on the other hand, can function as an inhibitor at the second stage, preventing the oxidation of dopa quinone. It can also keep fully formed melanin in the reduced, light-colored form.[33, 144]

If the oxidation of tyrosine is inhibited at the first stage of the reaction, the colorless, granular, cytoplasmic particulates present in the melanocytes should not exhibit any of the properties of melanin. Such particles would have to be considered as protein aggregates and complexes that are products of the hyaline matrix of the cytoplasm. Potentially they may be capable of forming complexes with the polymers of melanin.[108] With a scheme that postulates inhibition of melanin synthesis at the outset of the reaction, it is difficult to account for the chemical properties exhibited by the colorless granules that are characteristic of the melanins.

If thiol inhibition does not occur in the initial stage of the reaction and the synthesis proceeds normally to 5,6 dihydroxyindole, colorless granules might also be formed. As long as oxidation to the quinone is prevented by a reducing agent, polymerization of the reduced indole derivative would result in colorless granules. The 5,6 dihydroxyindole compound is the precursor of melanin that cannot be isolated because it is too easily oxidized. Its methyl derivative, however, has been isolated in crystalline form.[139]

By observing young amelanotic melanocytes in tissue culture for several days, an increase in the polymerization of the granules was evident. The small, colorless granules in the finely dendritic cells became larger and coarser and took on a reddish-green iridescence. The presence of partially oxidized brown melanin in some of the melanocytes may be explained by the characteristic lack of uniformity of malignant cells in a tumor. There may be a variation in the number of reducing groups present. Oxidation of the leukogranules to a colored compound might also be a local phenomenon, depending upon the position of the melanocyte in the tumor mass. In this scheme of events, thiol groups would have the important function of keeping the polymerized 5,6 dihydroxyindole derivative in a reduced state. The color differences ordinarily found among melanins could be caused by different states of oxidation and polymerization of the same substance.

The last hypothesis seems to satisfy best the conditions observed in the melanocytes of amelanotic melanomas. Since labeled tyrosine is incorporated in the tumor cells, the pigment reaction is probably not blocked in the initial stage. Since the cells with brown granules are mainly mature cells, and since the cells with colorless granules are young proliferating melanocytes, a decoloration of completely formed pigmented granules does not seem possible. In such a situation, the color pattern would be reversed, since the older cells would be decolorized before the younger cells. Besides, such a decoloration was never observed in tissue culture. An increase in the size of colorless granules in maturing melanocytes has been observed, and it may be accounted for by the increased polymerization of the colorless dihydroxyindole derivative.

The organization of proliferating amelanotic melanocytes into nodules is typical of primary tumors that frequently show distinctive structural organization in tissue culture.[4] The difference in growth characteristics of explants from the same tumor may be explained on the basis of such discrete nodules or clumps of melanocytes scattered in the tumor mass.

COMPARISON OF AMELANOTIC MELANOCYTES WITH MELANOCYTES

Most amelanotic melanocytes are feebly dendritic and readily assume spherical or less defined shapes. This inability to maintain the specialized dendritic form may be a measure of anaplasia. The sparsity of mature, melanophorelike cells in amelanomas may be interpreted as the inability of amelanotic melanocytes to reach full maturity and differentiation. Amelanotic melanocytes grow more readily in tissue culture than typical melanocytes, and they have a greater incidence of nuclear aberrations, such as hyperchromatism, giant and bizarre-shaped nuclei, nucleolar enlargement, and multiple nucleoli. This is of interest in view of the diagnostic significance of nuclear changes in determining malignancy.

Melanin cannot be considered an important factor in tumor formation. Melanin is the normal metabolic product of pigment cells, and its absence in pigmented tumors is an indication of rapid growth and immaturity of the cells rather than of another tumor type. The amelanotic melanomas of albino hybrids show evidence of being relatively more neoplastic than the melanomas caused by the same genes in nonalbino or melanotic animals.

Summary

(1) Melanomas develop in platyfish-swordtail hybrids carrying macromelanophore genes of the platyfish in association with macromelanophore growth modifying genes of the swordtail. Similar macromelanophore factors, present in albino hybrids, are expressed in amelanotic melanomas. There is a difference in the *in vitro* proliferation of tumors that are obtained from different regions of the organism. The proliferation is generally related to the vascular supply *in vivo*.

(2) The melanocyte is the preponderant cell type in tissue cultures of fish melanomas. It is present in different stages of differentiation, depending upon the rapidity of growth of the particular tumor. Macromelanophores appear to be the terminal stage in melanocyte maturation. The melanocytes of amelanotic melanomas rarely reach the state of differentiation exhibited by some of the melanotic melanocytes.

(3) Melanogenic enzyme activity was present in the pigment cells of melanomas as well as in amelanotic melanomas. The albino gene appears to operate by means of reducing groups. Thiol groups were present in amelanotic melanocytes, but they could not be demonstrated in melanotic melanocytes. The hypothesis is advanced that pigment-granule synthesis follows a similar pattern in the amelanotic melanocytes as in the pigmented melanocytes until the 5,6 dihydroxyindole compound is formed. Oxidation to the quinone, which yields a pigmented product, may be inhibited in albino animals by thiol groups.

(4) The gray or black color macroscopically apparent in some of the tumors of albino hybrids is caused by the grouping of brown granular cells into dense masses, which results in an over-all black effect. The pigment has the chemical properties characteristic of melanin.

(5) Cytological characteristics of the neoplastic process are more prominent in amelanotic melanoma pigment cells than in the pigment cells of typical melanomas. The 4 color variations of xiphophorin melanomas are actually expressions of a single tumor. Melanin, per se, does not appear to be a significant factor in the tumor genesis.

(6) The hyperplastic epidermis associated with the pigment-cell

tumors grows readily in culture and exhibits characteristics of invasive cells. The epithelial cells frequently contain melanin granules and have melanogenic enzyme activity. The refractile inclusions present in the epithelial cells were identified as prekeratin and keratin.

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References

1. GORDON, M. 1948. Effects of five primary genes on the site of melanomas in fishes and the influence of two color genes on their pigmentation. Spec. Publ. N. Y. Acad. Sci. **4**: 216-268.
2. GRAND, C. G., M. GORDON & G. CAMERON. 1941. Cell types in tissue culture of fish melanotic tumors compared with mammalian melanomas. Cancer Research. **1**: 660-666.
3. LEVINE, M. 1948. The cytology of the typical and the amelanotic melanoma. Spec. Publ. N. Y. Acad. Sci. **4**: 177-215.
4. CAMERON, G. 1950. Tissue Culture Technique. Academic Press. New York, N. Y.
5. RAFALKO, J. S. 1946. A modified Feulgen technique for small and diffuse chromatin elements. Stain Technol. **21**: 91-93.
6. LESSLER, M. A. 1948. Some quantitative aspects of the Feulgen reaction for DNA. Science. **108**: 419-420.
7. BRACHET, J. 1953. The use of basic dyes and ribonuclease for the cytochemical detection of ribonucleic acid. Quart. J. Microscop. Sci. **94**: 1-10.
8. KAY, E. R. M. 1953. A mixture of methyl green and pyronin for cell fractionation studies. Stain Technol. **28**: 41-44.
9. KURNICK, N. B. 1949. Methyl green-pyronin 1. Basis of selective staining of nucleic acids. J. Gen. Physiol. **33**: 243-264.
10. KURNICK, N. B. 1950. The quantitative estimation of desoxyribosenucleic acid based on methyl green staining. Exp. Cell Research. **1**: 151-158.
11. KURNICK, N. B. 1952. Histological staining with methyl green-pyronin. Stain Technol. **27**: 233-242.
12. POLLISTER, A. W. & C. LEUCHTENBERGER. 1949. The nature of the specificity of methyl green for chromatin. Proc. Natl. Acad. Sci. **35**: 111-116.
13. TAFT, E. B. 1951. The problem of a standardized technique for the methyl green-pyronin stain. Stain Technol. **26**: 205-212.
14. JACOBSON, W. & M. WEBB. 1951. The two types of nucleic acid during mitosis. J. Physiol. **112**: 2P.
15. JACOBSON, W. & M. WEBB. 1952. Two types of nucleoproteins during mitosis. Exp. Cell Research. **3**: 163-183.
16. SCHNEIDER, W. C. 1945. Phosphorus compounds in animal tissues. Extraction and estimation of desoxyribonucleic acid and of ribonucleic acid. J. Biol. Chem. **161**: 293-303.
17. PEARSE, A. G. E. 1953. Histochemistry. Little, Brown. Boston, Mass.
18. GLICK, D. 1949. Techniques of histo- and cytochemistry. Interscience. New York.

19. Cowdry, E. V. 1952. Laboratory techniques in biology and medicine. 3rd ed. Williams and Wilkins. Baltimore, Md.
20. Steedman, H. F. 1950. Alcian blue 8GS: A new stain for mucin. Quart. J. Microscop. Sci. **91**: 477-479.
21. McClung, C. E. 1939. Microscopical technique. **146**: 2nd ed. Hoeber. New York, N. Y.
22. Cain, A. J. 1949. On the significance of the plasmal reaction. Quart. J. Microscop. Sci. **90**: 75-86.
23. Cain, A. J. 1949. A critique of the plasmal reaction, with remarks on recently proposed techniques. Quart. J. Microscop. Sci. **90**: 411-425.
24. Danielli, J. F. 1949. Aldehydes in relation to absorption of fat from the intestine and metabolism of fat in the liver. Quart. J. Microscop. Sci. **90**: 309-315.
25. Danielli, J. F. 1949. A critical study of techniques for the cytochemical demonstration of aldehyde. Quart J. Microscop. Sci. **90**: 67-74.
26. Deane, H. W. & J. S. Andrews. 1953. A comparison of the staining of lipid droplets in the mouse ovary by the Schiff reaction and by the Ashbel-Seligman carbonyl reaction. J. Histochem. Cytochem. **1**: 283-295.
27. Menschik, Z. 1953. Nile blue histochemical method for phospholipids. Stain Technol. **28**: 13-18.
28. Baker, J. R. 1946. The histochemical recognition of lipine. Quart. J. Microscop. Sci. **87**: 441-470.
29. Ackerman, G. A. 1952. A modification of the Sudan black B technique for the possible cytochemical demonstration of masked lipids. Science. **115**: 629-631.
30. Tandler, C. J. 1954. An argentaffin component of the nucleolus. J. Histochem. Cytochem. **2**: 165-166.
31. Lerner, A. B., T. B. Fitzpatrick, E. Calkins & W. H. Summerson. 1949. Mammalian tyrosinase: preparation and properties. J. Biol. Chem. **178**: 185-195.
32. Lerner, A. B., T. B. Fitzpatrick, E. Calkins & W. H. Summerson. 1951. Mammalian tyrosinase: action on substances structurally related to tyrosine. J. Biol. Chem. **191**: 799-806.
33. Lerner, A. B. 1953. Metabolism of phenylalanine and tyrosine. Advances in Enzymol. **14**: 73-128.
34. Van Duijn, P. 1953. Inactivation experiments on the dopa factor. J. Histochem. Cytochem. **1**: 143-150.
35. Barrnett, R. J. & A. M. Seligman. 1952. Histochemical demonstration of protein-bound sulfhydryl groups. Science. **116**: 323-327.
36. Worley, L. G. & H. W. Spater. 1952. The cytoplasmic cytology of sarcoma 180. Quart. J. Microscop. Sci. **93**: 413-425.
37. Gordon, M. 1953. Preface. Pigment Cell Growth. Acad. Press. New York, N. Y.
38. Fitzpatrick, T. B. & A. B. Lerner. 1953. Terminology of pigment cells. Science. **117**: 640.
39. Grand, C. G. 1938. Clasmatosis in the melanoblast. Am. J. Cancer. **33**: 394-400.
40. Marcus, T. R. & M. Gordon. 1954. Transplantation of the Sc melanoma in fishes. Zoologica. **39**: 123-131.
41. Gordon, M. 1951. Genetic and correlated studies of normal and atypical pigment cell growth. Growth. **10**: 153-219.
42. Holtfreter, J. 1947. Observations on the migration, aggregation, and phagocytosis of embryonic cells. J. Morphol. **80**: 25-55.
43. Algard, F. T. 1953. Morphology and migratory behavior of embryonic pigment cells studied by phase microscopy. J. Exptl. Zool. **123**: 499-522.
44. Grand, C. G., R. Chambers & G. Cameron. 1935. Neoplasm Studies 1. Cells of melanoma in tissue culture. Am. J. Cancer. **24**: 36-50.
45. Dalton, A. J., H. Kahler, M. J. Striebich & B. Lloyd. 1950. Finer structure of hepatic, intestinal and renal cells of the mouse as revealed by the electron microscope. J. Nat. Cancer Inst. **11**: 439-461.

46. DALTON, A. J. 1951. Electron micrography of epithelial cells of the gastro-intestinal tract and pancreas. Am. J. Anat. **89**: 109-133.
47. PALADE, G. E. & K. R. PORTER. 1952. The endoplasmic reticulum of cells *in situ*. Anat. Record. **112**: 370.
48. LOUSTALOT, P., G. H. ALGIRE, F. Y. LEGALLAIS & B. F. ANDERSON. 1952. Growth and histopathology of melanotic and amelanotic derivatives of the Cloudman melanoma S91. J. Natl. Cancer Inst. **12**: 1079-1118.
49. BREIDER, H. 1938. Die genetischen, histologischen, und zytologischen Grundlagen der Geschwulstbildung nach Kreuzung verschiedener Rassen und Arten lebendgebarender Zahnkarpfen. Z. Zellforsch. **28**: 784-828.
50. ERMIN, R. & M. GORDON. 1955. Regeneration of melanomas in fishes. Zoologica. **40**: 53-83.
51. SPARROW, A. H. & M. R. HAMMOND. 1947. Cytological evidence for the transfer of desoxyribose nucleic acid from nucleus to cytoplasm in certain plant cells. Am. J. Botany. **34**: 439-445.
52. THORELL, B. 1944. Behavior of the nucleolar apparatus during growth and differentiation of the normal blood cells in the adult stage. Acta Med. Scand. **117**: 334-375.
53. LUDFORD, R. J. & J. SMILES. 1953. Malignant cell polymorphism studied by phase contrast microscopy. J. Roy. Microscop. Soc. **72**: 149-154.
54. LUDFORD, R. J. 1954. Nuclear structure and its modifications in tumors. Brit. J. Cancer. **8**: 112-131.
55. CASPERSSON, T. & J. SCHULTZ. 1940. Ribonucleic acids in both nucleus and cytoplasm, and the function of the nucleolus. Proc. Nat. Acad. Sci. **26**: 507-515.
56. LILLIE, R. D. 1948. Histopathologic Technique. Blakiston. Philadelphia, Pa.
57. CAIN, A. J. 1950. The histochemistry of lipoids in animals. Biol. Revs. Cambridge Phil. Soc. **25**: 73-112.
58. PORTER, K. R. 1953. Observations on a submicroscopic basophilic component of cytoplasm. J. Exptl. Med. **97**: 727-750.
59. LILLIE, R. D. 1952. Ethylenic reaction of ceroid with performic acid and Schiff reagent. Stain Technol. **27**: 37-45.
60. JONES, O. P. 1947. Mitochondria and their relation to the so-called hyaloplasm. J. Lab. Clin. Med. **32**: 700-719.
61. BOURNE, G. 1935. Mitochondria, Golgi apparatus and vitamins. Australian J. Exptl. Biol. Med. Sci. **13**: 239-249.
62. ENDICOTT, K. M. & R. D. LILLIE. 1944. Ceroid, the pigment of dietary cirrhosis of rats. Am. J. Pathol. **20**: 149-153.
63. ENDICOTT, K. M. 1944. Similarity of the acid-fast pigment ceroid and oxidized unsaturated fat. Arch. Pathol. **37**: 49-53.
64. LEE, C. S. 1950. Histochemical studies of the ceroid pigment of rats and mice and its relation to necrosis. J. Nat. Cancer Inst. **11**: 339-349.
65. DEROBERTIS, E. D. P., W. W. NOWINSKI & F. A. SAEZ. 1954. General Cytology, 2nd ed. Saunders. Philadelphia, Pa.
66. NORMAN, C. 1954. Quantitative study of distribution of sulfhydryl groups in the developing grasshopper *(Melanoplus differentialis)* embryo. Physiol. Zoöl. **27**: 141-156.
67. YAO, T. 1949. Cytochemical studies on the embryonic development of *Drosophila melanogaster*. 1. Protein sulfhydryl groups and nucleic acids. Quart. J. Microscop. Sci. **90**: 401-409.
68. MASSON, P. 1948. Pigment cells in man. Spec. Publ. N. Y. Acad. Sci. **4**: 15-51.
69. FITZPATRICK, T. B., A. B. LERNER, E. CALKINS & W. H. SUMMERSON. 1950. Occurrence of tyrosinase in horse and fish melanomas. Proc. Soc. Exptl. Biol. Med. **75**: 394-398.
70. MILLOTT, N. & W. G. LYNN. 1954. The effect of phenylthiourea on pigmentation by melanin in the developing frog, *Eleutherodactylus martinicensis*. Quart. J. Microscop. Sci. **95**: 17-22.
71. KULL, F. C., R. BONORDEN & R. L. MAYER. 1954. Inhibition of melanin

formation *in vivo* by 4-chlororesorcinol. Proc. Soc. Exptl. Bio. Med. **87**: 538-540.

72. ROTHMAN, S. 1940. Oxidation of tyrosine by ultraviolet light in its relation to human pigmentation. Proc. Soc. Exptl. Biol. Med. **44**: 485-486.
73. LERNER, A. B. & T. B. FITZPATRICK. 1950. Mammalian melanin formation: 1. Biochemical Studies. Zoologica. **35**: 27.
74. HESSELBACH, M. L. 1953. Control of melanization of S91 tumors by selective transfer, and biochemical studies of the tumors produced. Pigment Cell Growth. Acad. Press. New York, N. Y. : 189-210.
75. WEISS, P. 1944. *In vitro* transformation of spindle cells of neural origin into macrophages. Anat. Record. **88**: 205-221.
76. LEWIS, M. R. & W. H. LEWIS. 1924. Monocytes, macrophages, epithelioid cells and giant cells. Anat. Record. **29**: 391.
77. LEWIS, W. H. 1925. The transformation of mononuclear blood cells into macrophages, epithelioid cells, and giant cells. Harvey Lect. **21**: 77-112.
78. LONG, M. 1954. Progress report cytochemistry and biochemistry of differentiation in gynecological neoplasms. Col. Univ. Coll. Phys. and Surg. New York, N. Y. : 14-28.
79. BERENBLUM, I. 1954. A speculative review: the probable nature of promoting action and its significance in the understanding of the mechanism of carcinogenesis. Cancer Research. **14**: 471-477.
80. EWING, J. 1942. Neoplastic Diseases. 4th ed. Saunders. Philadelphia, Pa.
81. ERMIN, R. 1953. On a case of xantho-erythrophoroma formation in *Platypoecilus maculatus* var. fuliginosus. Rev. fac. sci. univ. Istanbul. Serie B. **18**: 301-314.
82. GOODRICH, H. B. & D. L. BIESINGER. 1953. The histological basis of color patterns in three tropical marine fish with observations on regeneration and the production of the blue color. J. Morphol. **93**: 465-487.
83. LUDFORD, R. J. 1925. The general and experimental cytology of cancer. J. Roy. Microscop. Soc. 249-292.
84. HORNING, E. S. & K. C. RICHARDSON. 1930. Cytological differences between normal and malignant cells. Med. J. Australia. **17**: 238-246.
85. COWDRY, E. V. 1940. Properties of cancer cells. Arch. Pathol. **30**: 1245-1274.
86. SPITZ, S. 1948. Melanomas of childhood. Am. J. Pathol. **24**: 591-609.
87. SOUTHAM, C. M. & P. J. GOETTLER. 1953. Growth of human epidermoid carcinoma cells in tissue culture. Cancer. **6**: 809-826.
88. DEDERER, P. H. 1921. The behavior of cells in tissue cultures of *Fundulus heteroclitus* with special reference to the ectoderm. Biol. Bull. **41**: 221-240.
89. ZOLLINGER, H. U. 1948. Cytologic studies with the phase microscope. I. The formation of blisters on cells in suspension (potocytosis), with observations on the nature of the cellular membrane. Am. J. Pathol. **24**: 545-558.
90. YOUNG, J. Z. 1953. Intracellular membranes. Quart. J. Microscop. Sci. **94**: 399-406.
91. ZOLLINGER, H. U. 1948. Cytologic studies with the phase microscope. II. The mitochondria and other cytoplasmic constituents under various experimental conditions. Am. J. Pathol. **24**: 569-589.
92. SJÖSTRAND, F. S. 1953. Electron microscopy of mitochondria and cytoplasmic double membranes. Nature. **171**: 31-32.
93. SJÖSTRAND, F. S. & J. RHODIN. 1953. The ultrastructure of the proximal convoluted tubules of the mouse kidney as revealed by high resolution electron microscopy. Exptl. Cell Research. **4**: 426-456.
94. BRADFIELD, J. R. G. 1950. The localization of enzymes in cells. Biol. Revs. Cambridge Phil. Soc. **25**: 113-157.
95. STOWELL, R. E. 1952. Use of histochemical and cytochemical techniques in problems in pathology. Lab. Invest. **1**: 210-230.
96. ACKERMAN, G. A., R. A. KNOUFF & H. A. HOSTER. 1951. Cytochemistry and morphology of neoplastic and nonneoplastic human lymph node cells

with special reference to Hodgkin's disease. J. Natl. Cancer Inst. 12: 465-489.

97. HULL, T. C. 1953. Some observations on the histochemical composition of the Yoshida rat ascites sarcoma. Proc. Am. Assoc. Cancer Res. 1: 23.
98. CHÈVREMONT, M. & H. FIRKET. 1953. Alkaline phosphatase of the nucleus. Intern. Rev. Cytol. 2: 261-288.
99. SULKIN, N. M. & J. H. GARDNER. 1948. The acid and alkaline phosphatase activity in the normal and recovering liver of the rat. Anat. Record. 100: 143-157.
100. BAHR, G. F. 1954. Osmium tetroxide and ruthenium tetroxide and their reactions with biologically important substances. Exptl. Cell Research. 7: 457-479.
101. COWDRY, E. V. 1932. Special Cytology. 2nd ed. Hoeber. New York, N. Y. : 3-38.
102. LITVAC, A. 1939. Sur la kératinisation epitheliale *in vitro*. Arch. D'Anat. Micro. 35: 55-63.
103. THOMPSON, E. O. P. 1954. Modification of tyrosine during performic acid oxidation. Biochem. Biophys. Acta. 15: 440-441.
104. TOENNIER, G. & R. P. HOMILLER. 1942. The oxidation of amino acids by hydrogen peroxide in formic acid. J. Am. Chem. Soc. 64: 3054-3056.
105. BURK, D., G. H. ALGIRE, M. HESSELBACH, C. E. FISCHER & F. Y. LEGALLAIS. 1948. Tissue metabolism of transplanted mouse melanomas, with special reference to characterization by paraphenylenediamine. Spec. Publ. N. Y. Acad. Sci. 4: 437-446.
106. DU BUY, H. G., M. W. WOODS & J. L. SHOWACRE. 1953. Glycolytic activities of slices and subcellular fractions of the Cloudman S91 mouse melanoma compared to a transplantable hepatoma and normal liver. Pigment Cell Growth. Acad. Press. New York, N. Y. : 335-351.
107. GIROUD, A. & C. P. LEBLOND. 1951. The keratinization of epidermis and its derivatives, especially the hair, as shown by X-ray diffraction and histochemical studies. Ann. N. Y. Acad. Sci. 53: 613-626.
108. KOPAC, M. J. 1948. Some cytochemical aspects of pigmented cells. Spec. Publ. N. Y. Acad. Sci. 4: 423-432.
109. SMITH, D. T. 1920. The pigmented epithelium of the embryo chick's eye studied *in vivo* and *in vitro*. Bull. Johns Hopkins Hosp. 31: 239-246.
110. LAXER, G., J. SIKORSKI, C. S. WHEWELL & H. J. WOODS. 1954. Electron microscopy of melanin granules isolated from pigmented mammalian fibers. Biochem. Biophys. Acta. 15: 174-185.
111. PLUMMER, J. I. & M. J. KOPAC. 1953. The *in vitro* production of pigment granules. Pigment Cell Growth. Acad. Press. New York, N. Y. : 305-317.
112. MEIROWSKY, E., L. W. FREEMAN & R. B. FISCHER. 1950. Observations on the structure, derivation and nature of melanin. Zoologica. 35: 29-30.
113. MEIROWSKY, E., L. W. FREEMAN & B. A. WISEMAN. 1951. Observations on melanization in isolated hair cells. Acta Dermato-Venereol. 31: 722-728.
114. WOODS, M. W., H. G. DUBUY, D. BURK & M. HESSELBACH. 1949. Cytological studies on the nature of the cytoplasmic particulates in the Cloudman S91 mouse melanoma, the derived Algire S91A partially amelanotic melanoma, and the Harding-Passey mouse melanoma. J. Natl. Cancer Inst. 9: 311-324.
115. DU BUY, H. G., M. W. WOODS, D. BURK & M. D. LACKEY. 1949. Enzymatic activities of isolated amelanotic and melanotic granules of mouse melanomas and a suggested relationship to mitochondria. J. Natl. Cancer Inst. 9: 325-335.
116. WOODS, M., H. DUBUY & D. BURK. 1950. Evidence for the mitochondrial nature and function of melanin granules. Zoologica. 35: 30-31.
117. DALTON, A. J. & M. D. FELIX. 1953. Phase contrast and electron micrography of the Cloudman S91 mouse melanoma. Pigment Cell Growth. Acad. Press. New York, N. Y. : 267-276.
118. SMITH, D. T. 1925. Evidence showing the existence of two distinct types

of pigment cells capable of giving rise to melanotic tumors. Bull. Johns Hopkins Hosp. **36**: 185-198.

119. GATES, R. R. 1942. Nucleoli and related nuclear structures. Botan. Rev. **8**: 337-409.
120. BECKER, W. S., T. B. FITZPATRICK & H. MONTGOMERY. 1952. Human melanogenesis. Cytology and histology of pigment cells (melanodendrocytes). Arch. Dermatol. and Syphilol. **65**: 511-523.
121. KULL, F. C., M. R. GRIMM & R. L. MAYER. 1954. Studies on inhibition of tyrosinase. Proc. Soc. Exptl. Biol. Med. **86**: 330-332.
122. ZIMMERMAN, A. A. & T. CORNBLEET. 1948. The development of epidermal pigmentation in the Negro fetus. J. Invest. Dermatol. **11**: 383-395.
123. MATSUMOTO, S. 1918. Demonstration of epithelial movement by the use of vital staining, with observations on phagocytosis in the corneal epithelium. J. Exptl. Zool. **27**: 37-47.
124. SMITH, D. T. 1921. The ingestion of melanin pigment granules by tissue cultures. Johns Hopkins Hosp. Bull. **32**: 240-244.
125. PARSHLEY, M. S. & H. S. SIMMS. 1950. Cultivation of adult skin epithelial cells (chicken and human) *in vitro*. Am. J. Anat. **86**: 163-183.
126. ALLEN, A. C. & S. SPITZ. 1954. Histogenesis and clinicopathologic correlation of nevi and malignant melanomas. Arch. Dermatol. and Syphilol. **69**: 150-171.
127. OPPENHEIMER, J. M. 1950. Atypical pigment cell differentiation in embryonic teleostean grafts and isolates. Zoologica. **35**: 22-23.
128. LERNER, A. B. & T. B. FITZPATRICK. 1950. Biochemistry of melanin formation. Physiol. Revs. **30**: 91-126.
129. FITZPATRICK, T. B., S. W. BECKER, A. B. LERNER & H. MONTGOMERY. 1950. Tyrosinase in human skin: demonstration of its presence and of its role in human melanin formation. Science. **112**: 223-225.
130. FIGGE, F. H. J. 1941. Effect of glutathione on tyrosinase and the significance of the dopa reaction. Proc. Soc. Exptl. Biol. Med. **46**: 269-272.
131. KERTÉSZ, D. 1954. The phenol-oxidizing enzyme system of human melanomas; substrate specificity and relationship to copper. J. Natl. Cancer Inst. **14**: 1081-1091.
132. FIGGE, F. H. J. 1938. Inhibition of tyrosinase melanin formation by sodium benzenone indophenol. Proc. Soc. Exptl. Biol. Med. **39**: 569-571.
133. FIGGE, F. H. J. 1940. Pigment metabolism studies. J. Comp. Cell. Physiol. **15**: 233-247.
134. GOODRICH, H. B. 1933. One step in the development of hereditary pigmentation in the fish *Oryzias latipes*. Biol. Bull. **65**: 249-252.
135. GREENSTEIN, J. P. & G. H. ALGIRE. 1944. Comparative oxidase activity of melanotic and amelanotic melanomas. J. Natl. Cancer Inst. **5**: 35-38.
136. HESSELBACH, M. L., M. WOODS & D. BURK. 1950. Oxidation activities of mouse melanomas with reference to melanization. Zoologica. **35**: 31.
137. FIGGE, F. H. J. 1948. Factors regulating the formation and the physical and chemical properties of melanin. Spec. Publ. N. Y. Acad. Sci. **4**: 405-422.
138. FLESCH, P. & S. ROTHMAN. 1948. Role of sulfhydryl compounds in pigmentation. Science. **108**: 505-506.
139. RAPER, H. S. 1927. The tyrosinase-tyrosine reaction. Biochem. J. **21**: 89-96.
140. RAPER, H. S. 1928. The aerobic oxidases. Physiol. Revs. **8**: 245-282.
141. MASON, H. S. 1953. The structure of melanins. Pigment Cell Growth. : 277-303. Acad. Press. New York, N. Y.
142. LERNER, A. B. & T. B. FITZPATRICK. 1953. The control of melanogenesis in human pigment cells. Pigment Cell Growth. : 319-333. Acad. Press. New York, N. Y.
143. ROTHMAN, S. 1942. *In vitro* studies on pigmentation. J. Invest. Dermatol. **5**: 61-67.
144. DENTON, C., A. B. LERNER & T. B. FITZPATRICK. 1952. Inhibition of melanin formation by chemical agents. J. Invest. Dermatol. **18**: 119-135.

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